Universidad Autónoma de Madrid
facultad de medicina
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Madrid, 2009

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This thesis has been completed at Instituto de Investigaciones Biomédicas “Alberto Sols” CSIC-UAM, Madrid during 2005-2009, with an initial financial support by Universidad Autónoma de Madrid (*Ayuda de Tercer Ciclo*) and a four-year fellowship by Ministerio de Ciencia e Innovación (*Formación de Profesorado Universitario*).

A mis padres,

a mi hermano.

A Alex

AGRADECIMIENTOS

Como se suele decir hay decisiones que te cambian la vida. Hace ahora cuatro años decidí cambiar la mía. Dejé mi querida Asturias y me vine a la capital en busca de una Tesis. Durante este viaje he pasado momentos buenos y malos, alegrías y penas, pero siempre por suerte me he encontrado con mucha gente que me ha acompañado durante este recorrido y con la que he compartido más o menos momentos y a los que hoy quiero darles las gracias.

En primer lugar darle las gracias al Prof. Alberto Muñoz, que amablemente me acogió hace cuatro años en su laboratorio, a pesar de que en aquel entonces no sobraba espacio. Gracias por saber llevarme y aguantar mis pequeñas "borderías" durante estos años de Tesis. Gracias también por darme la oportunidad de crecer personal y científicamente y conseguir hacer un trabajo del que me siento realmente orgullosa.

De la misma manera quiero agradecer al Prof. Carlos López Otín que una mañana que entré en su despacho buscando consejo me atendió amablemente y me guió hasta donde hoy me encuentro. Ese fue el inicio de mi viaje y hoy vemos que como todas las buenas decisiones ha dado buenas recompensas. Gracias también por ser un referente para todos los jóvenes de este país que deciden embarcarse en el duro camino de la Ciencia y ven en ti un ejemplo a seguir (ese, al menos, ha sido siempre mi motor).

De manera especial quiero darle las gracias a la Dra. Valle. Noe, sin ti esta Tesis no habría sido posible, ahora ya tienes una Tesis y media! Gracias por haber estado a mi lado en mis primeros pasos en el laboratorio y enseñarme tantas cosas y planificarlas todas a la vez (lo rápido que hacíamos experimentos!). Gracias por haber sido compañera, amiga, hermana y casi hasta madre y sobre todo porque tú si que has tenido que aguantar mis cambios de humor y mis "cositas" difíciles.

Durante este periodo hemos colaborado con mucha gente que ha hecho posible que este trabajo saliera adelante, a todos ellos muchas gracias. Quiero destacar al Dr. Félix Bonilla y a los miembros de su laboratorio del Hospital Puerta de Hierro de Madrid, especialmente a José Miguel y a Cristina, por todas esas RT-PCRs que van en esta Tesis y son vuestras. Gracias a la Dra. Aurora Astudillo y Marta Sánchez Pitiot del Servicio de Anatomía Patológica del Hospital Universitario Central de Asturias por ayudarnos con las inmunohistoquímicas. Agradecer también al laboratorio del Prof. Carlos López Otín, por acogerme en mi pequeña estancia allí. En especial gracias al Dr. José María Pérez Freije, por su ayuda y consejos científicos durante esta Tesis, y por supuesto por haber clonado la cistatina D! Gracias a Victor Quesada por su ayuda en los experimentos de actividad enzimática y a Ignacio Varela por preocuparse de que tuviéramos de todo.

No me puedo olvidar de mis compañeros durante mi estancia en Nueva York. En primer lugar gracias a la Dra. Eva Hernando, por acogerme en su laboratorio, por preocuparse de que todo estuviera bien y sobre todo por ser una excelente persona. Gracias a la pequeña "comunidad del Tupper": Miguel, María, Olga, Mar, Alicia, Sergi… por esas estupendas horas de la comida y esos grandiosos *cookies days*. Gracias especialmente a Miguel por ocuparte de mi en el Smilow 305, por tu humor, por ayudarme con mi trabajo y enseñarme cosas nuevas. Mary, gracias por ser como eres, que siempre se puede contar contigo, ya sea en una *roof night* (qué éxito tuvimos!) o tomando unas cañitas en el Quevedo. Olga, la mejor *new yorker* sin duda alguna, gracias por ser una persona extraordinaria, por conocer tantas cosas de NYC y llevar siempre contigo la cámara de fotos! María, gracias por tu alegría y por ser tan de Madriz! Laura *New York, New York* , eres estupenda, gracias por haber sido mi compañera de turisteo, que bueno haber coincido! Lorena, mi "relevo" en NYU, que pena que solo coincidimos unas semanitas, nos lo habríamos pasado genial! *I cannot forget mentioning Laura, Doug and Avital. Thank you guys for your help trying to make my stay there much nicer. Laura thank you for your endless smile, you are so enthusiastic! Doug, thank you for trying to speak with me everyday, I know it was a very difficult work! Avital you are so sweet, thank you very much for your help and your eternal patience with my shaky English! Thanks also to*

Silvia and Martha for help us in our daily work in the lab. En definitiva que habría sido de mi en la Gran Manzana sin todos vosotros!!

Por supuesto, mención especial se merecen mis compañeros del 231, que han sido mi pequeña familia durante estos años. Gracias a Teresa y Diego por hacer que en el laboratorio nunca faltara de nada. Gracias José por compartir tus conocimientos sobre clonajes conmigo y Oscar por ayudarnos al inicio de nuestros ChIPs. Gracias Pepa por ser como un libro abierto y ayudarme siempre que te he pedido consejo (que no han sido pocas veces). Gracias Toño por tus historias, por tu risa contagiosa, por ser un gran compañero. Paloma, eres la alegría del laboratorio, gracias por ser tan positiva y ver siempre el lado bueno de las cosas. Gracias a Diego por haber sido el mejor compañero en los cursos de doctorado y de escalada, y casi casi el mejor compañero de poyata (igualado con Noe), gracias por haberme escuchado siempre y darme tan buenos consejos. Gracias Chus porque contigo es un gusto trabajar y hablar de ciencia, eres el alma del 231, gracias por tus innumerables consejos y sobretodo porque eres una persona estupenda, de esas de las que es difícil encontrar. Gracias Fabio por haber venido al laboratorio, por hacerme tanta compañía, por tus chocolatitos y tus regalitos y por nuestras conversaciones "científicas" o no. No me puedo olvidar de las chicas que nos habéis abandonado. Laura, gracias por ser una gran persona y estar dispuesta a ayudar siempre y por todos tus consejos sobre cremitas! Noe, gracias por haber compartido poyata, reactivos y experimentos, también agradecerte tu excelente labor como monitora de aventuras del labo 231, por habernos hecho pasar esos buenos momentos y por haberme iniciado en la escalada! Nati, gracias por esos tres años de Tesis conmigo, has sido una maravillosa compañera, siempre con tu risa y tus comentarios picantes, pero sobretodo gracias por haber sido una buena amiga, por lo bien que nos lo hemos pasado, con o sin cerveza, por apoyarnos y entendernos. Gracias también a José, mi compañero de escritura de Tesis, que aunque oficialmente seas del 251 "extraoficialmente" eres uno más de los nuestros. Gracias por tu inestimable ayuda durante estos meses de escritura, por hacer que no se me olvide ni un solo detalle, por ser una bella persona, por compartir tu música conmigo e intentar que aprendiera a tocar la guitarra (misión imposible, te lo dije!).

Son muchas las personas que hacen que el IIBM funcione y podamos hacer nuestro trabajo, a todos ellos gracias. Sobre todo gracias al servicio de secuenciación, Gemma, Conchi, Diego, Diana y Eva por vuestro ayuda con secuencias y RT-PCRs; y al servicio de Imagen, Ricardo, Javier y Antonio, por vuestra ayuda durante la impresión de esta Tesis. También agradecer a Ricardo Ramos del Parque Científico por su ayuda en la validación por RT-PCR, y a la Dra. Encarna Fermiñan y el Dr. Javier de las Rivas, del Centro de Investigación del Cáncer de Salamanca por su ayuda con los *microarrays*.

Gracias a mis compañeros de carrera, donde se empezó a gestar todo. Gracias sobre todo a Marina, porque las dos iniciamos esta aventura en Madrid juntas, por haber sido mi compañera durante casi dos años y haber vivido muy buenos momentos. En especial quiero darle las gracias a todos mis amigos de tierras madrileñas, a los que habéis compartido conmigo algún momento de estos cuatro años y habéis hecho que la vida fuera del laboratorio sea más divertida: Bosco, Alberto, Rafa, Luis, Paola, Cirilo, Manu, Rocío, Miguel, Ruso, Yubero, Marta, Germán, Anita, Yacob, Meno, Ewelina, Ana, Iván, Javi, Megan, Carlos, Lidia, Jesús… sois muchos y se que me dejaré algún nombre en el tintero, pero ya sabéis que estáis aquí incluidos.

Gracias a María, Ángel y Ricardo, mi "familia" de Zaragoza, por acogerme tan bien siempre que estoy por allí, aunque no vayamos tanto como a vosotros os gustaría.

Quiero dejar lo más importante para el final, a los que sois una parte vital de mi. En primer lugar gracias a mis amigos de Asturias, Nacho, Marta, Alberto, Adrián, Andrés… Y en especial gracias a María, Irene y Jero, por haber estado a mi lado durante tantos años, por haber compartido tantas cosas juntos, porque sin vosotros no hubiera llegado a donde estoy ahora y porque a pesar de estar lejos seguimos manteniendo la misma amistad.

En segundo lugar gracias a mi familia, primos, tíos y demás que siempre estáis ahí, sobre todo gracias a mi madrina Mari, a mi prima Inma y a mi tía Rosa Mari. Mención especial para mi tía María Adelia, que durante

tantos años has cuidado de nosotros y te has preocupado de que todo estuviera bien. Gracias por estar cuando más lo necesitábamos. Pero sobre todo quiero darle las gracias a mi hermano José, hemos estado juntos durante 28 (casi 29 ya) años, sin ti no hubiera salido adelante, has cuidado de mi desde muy pequeña y has sido mi apoyo en tantos momentos difíciles. Has sido el mejor compañero de carrera que he tenido y sobre todo el mejor hermano que nadie se puede imaginar, muchísimas gracias por todo. Aquí también incluyo a Marta, mi cuñada preferida, porque ya son muchos años juntas, gracias por tu ayuda estos últimos meses en Oviedo y sobre todo gracias por hacer feliz a mi hermano. Gracias a mi padre, por haber cuidado de que sus hijos tuvieran lo que necesitaban, siento tanto no haber estado junto a ti tanto como debería en los últimos momentos, pero se que habrías estado muy orgulloso de mí por haber terminado esta Tesis. Finalmente gracias a mi madre, son ya tantos años sin que estés aquí, pero ni un solo momento me he olvidado de ti, espero haber cumplido parte de los sueños que tenías para tu hija. Esta Tesis va dedicada especialmente a ti.

Por último quiero darle las gracias a Alex, por estar conmigo estos dos últimos años, por apoyarme en todo lo que hago, por fascinarte por mi trabajo, por aguantarme día a día y hacer que mis días sean siempre felices y porque pase lo que pase a partir de ahora espero que lo pasemos juntos.

"If you want to be happy, be"

Leo Tolstoy

"Failure is the opportunity to begin again more intelligently"

Henry Ford

"Ciencia es desechar una bonita teoría por un asqueroso hecho"

Thomas Henry Huxley

ABSTRACT

Colorectal cancer (CRC) is one of the most common human neoplasias. Epidemiological and preclinical studies have shown that 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), the most active metabolite of vitamin D_3, has wide but not fully understood antitumor activity. Most, if not all, 1α,25$(OH)_2D_3$ actions are mediated by vitamin D receptor (VDR), a member of the nuclear receptor superfamily of transcription factors whose expression is lost during CRC progression. Cystatin D (*CST5* gene product) is an inhibitor of several cysteine proteases of the cathepsin family. Cystatin D has a more restricted pattern of tissue expression and narrower inhibitory profile than other members of the cystatin family, as well as an unknown biology. A previous transcriptomic analysis of 1α,25$(OH)_2D_3$ action on human colon cancer cells revealed *CST5* as a candidate target gene.

The results described in this Thesis show that 1α,25$(OH)_2D_3$ increases *CST5* RNA and protein levels in human CRC cells. Consistently, diminished cathepsin L activity was detected in 1α,25$(OH)_2D_3$-treated cells. 1α,25$(OH)_2D_3$ promotes VDR binding to, and transcriptional activation of, the *CST5* promoter. In cells lacking endogenous cystatin D, ectopic cystatin D expression inhibited cell proliferation, migration and anchorage-independent growth. Moreover, cystatin D antagonized the Wnt/β-catenin signaling pathway and repressed c-MYC expression. Additionally, cystatin D repressed the epithelial-mesenchymal transition inducers *SNAI1, SNAI2, ZEB1* and *ZEB2,* and, conversely, induced E-cadherin and other adhesion proteins. Transcriptomic analyses have identified a panel of candidate target genes whose RNA levels in CRC cells are modulated by cystatin D. Furthermore, ectopic cystatin D expression blunted xenograft tumor growth in immunodeficient mice. *CST5* knockdown using shRNA abrogated the antiproliferative effect of 1α,25$(OH)_2D_3$, attenuated E-cadherin expression, and increased c-MYC expression. Interestingly, mutant cystatin D proteins with reduced antiproteolytic activity preserve the antiproliferative but not the cell migration-inhibitory effects. In human CRC tumors, we found a strong correlation between the expression of VDR and E-cadherin and that of cystatin D. Additionally, the loss of cystatin D correlated with poor tumor differentiation. Our results show that *CST5* acts as a tumor suppressor gene with unpredicted effects that may contribute to the antitumor action of 1α,25$(OH)_2D_3$ in colon cancer.

RESUMEN

El cáncer colorrectal (CRC) es una de las neoplasias de mayor incidencia y mortalidad. Estudios epidemiológicos y preclínicos indican que la 1α,25-dihidroxivitamina D_3 (1α,25$(OH)_2D_3$), el metabolito más activo de la vitamina D_3, tiene una amplia pero no del todo conocida actividad antitumoral frente al CRC. La mayoría, si no todas, las acciones de la 1α,25$(OH)_2D_3$ están mediadas por el receptor de vitamina D (VDR), un factor de transcripción de la superfamilia de los receptores nucleares cuya expresión se pierde durante la progresión del CRC. La cistatina D (producto del gen *CST5)* es un inhibidor de varias cisteín–proteasas de la familia de las catepsinas. La cistatina D tiene un perfil más restringido de expresión tisular y un perfil de inhibición más limitado que otros miembros de la familia de las cistatinas, así como una biología poco conocida. Estudios transcriptómicos previos sugieren que *CST5* es un gen diana de la 1α,25$(OH)_2D_3$ en células humanas de cáncer colon.

Los resultados de esta Tesis indican que la 1α,25$(OH)_2D_3$ aumenta los niveles de RNA y proteína de *CST5* en células de CRC humano. Consecuentemente, en las células tratadas con 1α,25$(OH)_2D_3$ se detectó una disminución en la actividad de la catepsina L. La 1α,25$(OH)_2D_3$ promueve la unión de VDR al promotor del gen *CST5* y su activación transcripcional. En células que carecen de expresión endógena de cistatina D, su expresión ectópica inhibió la proliferación, migración y crecimiento independiente de anclaje a sustrato. Más aún, la cistatina D antagonizó la ruta de señalización Wnt/β-catenina y reprimió la expresión de *c-MYC*. Además, la cistatina D reprimió la expresión de los genes inductores de transición epitelio-mesénquima *SNAI1, SNAI2, ZEB1* y *ZEB2*, y, de manera opuesta, indujo la expresión de E-cadherina y otras proteínas de adhesión. Estudios transcriptómicos han identificado una serie de genes candidatos a ser regulados por cistatina D en células de CRC humano. *In vivo*, la expresión ectópica de cistatina D bloqueó el crecimiento de tumores xenotransplantados en ratones inmunosuprimidos. El silenciamiento del gen *CST5* utilizando shRNA inhibió los efectos antiproliferativos de la 1α,25$(OH)_2D_3$, atenuó la expresión de E-cadherina y aumentó la expresión de c-MYC. Versiones mutantes de cistatina D con reducida actividad antiproteolítica mantuvieron el efecto antiproliferativo pero no el inhibidor de migración celular. En tumores colorrectales humanos encontramos una fuerte correlación entre la expresión de VDR y E-cadherina y la de cistatina D. Además durante la progresión tumoral la pérdida de cistatina D se asocia con menor grado de diferenciación. Nuestros datos muestran que *CST5* actúa como un gen supresor tumoral que puede contribuir a la acción protectora de la 1α,25$(OH)_2D_3$ frente al cáncer de colon.

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ABBREVIATIONS

1α,25(OH)$_2$D$_3$: 1α,25-dihydroxyvitamin D$_3$

25(OH)D$_3$: 25-hydroxyvitamin D$_3$

9cRA: 9-*cis* retinoic acid

ACF: aberrant crypt foci

AF: activation function

APC: adenomatous polyposis coli

bHLH: basic helix-loop-helix

CDK: cyclin-dependent kinase

ChIP: Chromatin immnunoprecipitation

CIN: chromosomal instability

CK1: casein kinase 1

CRC: colorectal cancer

C_T: threshold cycle

CYP24: 24-hydroxylase

CYP27A1: vitamin D$_3$-25-hydroxylase

CYP27B1: 25(OH)D$_3$-1α-hydroxylase

DBD: DNA-binding domain

DBP: vitamin D-binding protein

DHA: docosahexaenoic acid

EMT: epithelial-mesenchymal transition

FAP: familial adenomatous polyposis

GAPDH: gliceraldehyde 3-phosphate deshydrogenase

GI: gastrointestinal

GSK: glycogen synthase kinase

HAT: histone acetyltransferase

HDAC: histone deacetylase

HNPCC: hereditary nonpolyposis colorectal cancer

HNSCC: head and neck squamous cell carcinoma

ISC: intestinal stem cell

LBD: ligand-binding domain

LEF: lymphoid enhancer-binding protein

LRP: lipoprotein receptor-related protein

Luc: *Firefly* luciferase

MET: mesenchymal-epithelial transition

MIN: microsatellite instability

MMR: mismatch repair

NICD: notch intracellular domain

PAI: plasminogen activator inhibitor

PBS: phosphate-buffered saline

PI: propidium iodide

PPAR: peroxisoma proliferator activated receptor

PTH: parathyroid hormone

RA: retinoic acid

RAR: retinoid acid receptor

RARE: retinoic acid response element

Rluc: *Renilla reniformes* luciferase

RUNX: runt-related transcription factor

RXR: retinoid X receptor

SDHA: succinate deshydrogenase complex, subunit A

SFRP: secreted Frizzled-related protein

shRNA: short hairpin RNA

TBP: TATA-binding protein

TCF: T-cell factor

TGF: transforming growth factor

TIMP: tissue inhibitor of metalloproteinases

TR: thyroid hormone receptor

tRA: *all*-trans retinoic acid

UBC: ubiquitin C

UVB: ultraviolet light

VDR: vitamin D receptor

VDRE: vitamin D response element

ZO: *zonula occludens*

INTRODUCTION

1. Vitamin D

1.1 Biochemistry

Vitamin D is a group of fat-soluble secosteroid prohormones (steroids in which one of the bonds in the steroid rings is broken), the two major forms are vitamin D_3 (or cholecalciferol), synthesized by vertebrates, and vitamin D_2 (or ergocalciferol), produced by invertebrates, fungi and plants.

Vitamin D_3 is naturally present in very few components of the human diet, and is also available as a dietary supplement, but this only contributes to 10% of total vitamin D_3 in the body. The 90% remaining is produced in the skin when 7-dehydrocholesterol reacts with ultraviolet (UVB) light (wavelengths between 270–300 nm), with peak synthesis occurring at 295-297 nm [212]. These wavelengths occur daily within the tropics, daily during the spring and summer seasons in temperate regions, and almost never within the arctic circles. After photolytic conversion of 7-dehydrocholesterol in previtamin D_3, this is spontaneously isomerized to vitamin D_3 that enters the circulation and is transported bound to the carrier plasma protein vitamin D-binding protein (DBP) [119] (Figure 1). Vitamin D_3 obtained from sun exposure, food, and supplements is biologically inert (prohormone) and must undergo two hydroxylations in the body for activation. First, vitamin D_3 is hydroxylated in the liver to 25-hydroxycholecalciferol ($25(OH)D_3$ or calcidiol, the major circulating form of vitamin D) by the enzyme vitamin D_3-25-hydroxylase (CYP27A1 and CYP2A) produced by hepatocytes, and stored until it is needed. Later, in the kidney, the $25(OH)D_3$-1α-hydroxylase enzyme (CYP27B1) catalyzes a second hydroxylation of $25(OH)D_3$, resulting in the formation of 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$ or calcitriol, the most potent form of vitamin D). Most of the physiological effects of vitamin D_3 in the body are related to the activity of $1\alpha,25(OH)_2D_3$ [121]. Although the kidney is the main responsible of the circulating $1\alpha,25(OH)_2D_3$ synthesis, CYP27B1 is also expressed in other tissues as skin, bone, prostate, breast, lung and colon, where $1\alpha,25(OH)_2D_3$ acts in an autocrine and/or paracrine manner [332].

The catabolism of $1\alpha,25(OH)_2D_3$ and its metabolites is mediated by the oxidation of its lateral chain, initiated by the hydroxylation of 24' position by ubiquitous 24-hydroxylase (CYP24). Subsequent oxidations generate metabolites less active till calcitroic acid, which is biologically inert and is excreted. The $1\alpha,25(OH)_2D_3$ plasma levels are tightly controlled by the regulation of CYP27B1 and CYP24 expression by parathyroid hormone (PTH) and $1\alpha,25(OH)_2D_3$ itself [109,199].

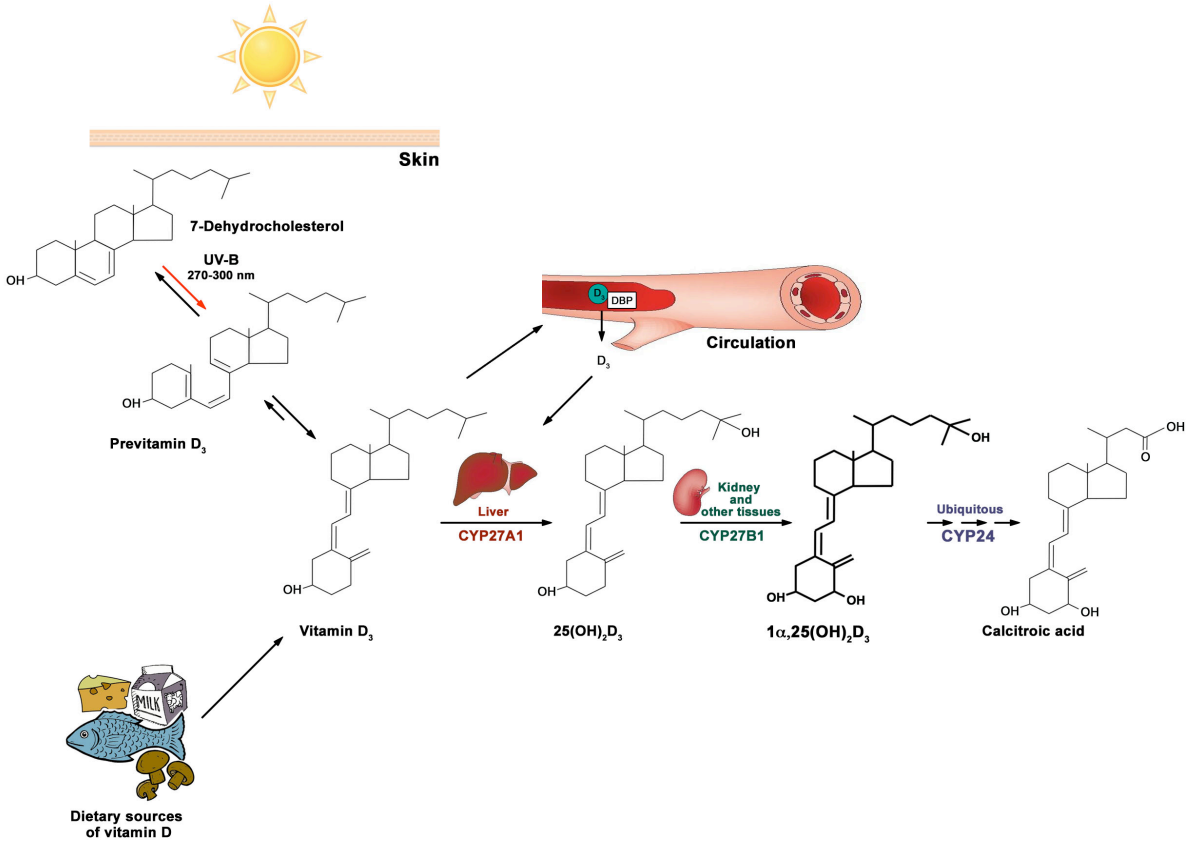

Figure 1. Metabolism of 1α,25(OH)$_2$D$_3$. Key enzymes and tissues involved in 1α,25(OH)$_2$D$_3$ synthesis and catabolism are shown.

1.2 Biological actions of 1α,25(OH)$_2$D$_3$

1α,25(OH)$_2$D$_3$ is a pleiotropic hormone with endocrine, paracrine and, probably, autocrine actions [256]. 1α,25(OH)$_2$D$_3$ is essential for promoting calcium absorption in the gut and maintaining adequate serum calcium and phosphate concentrations to enable normal mineralization of bone. It is also needed for bone growth and bone remodeling by osteoblasts and osteoclasts [120]. These are known as **classical actions** of vitamin D [44]. However, since 1981 **novel actions** including antiproliferative, pro-apoptotic and prodifferentiation effects in normal and tumor cells have been described [38,56,221]. In addition, numerous studies have reported the inhibition by 1α,25(OH)$_2$D$_3$ of tumor invasiveness and angiogenesis [45,110,221] and its importance as antimicrobial and immunomodulatory agent [10,177].

These novel 1α,25(OH)$_2$D$_3$ actions suggest its use for the treatment of hyperproliferative disorders such as psoriasis and cancer. However, the toxicity due to the hypercalcemic effect of 1α,25(OH)$_2$D$_3$ at therapeutic doses restricts the latter treatment. Many analogs (deltanoids) with less hypercalcemic effect are at different preclinical and clinical

development stages as single or combinatorial treatment against several neoplasias (www.clinicaltrials.gov) [56,62].

1.3 Mechanism of action of 1α,25(OH)$_2$D$_3$

1.3.1 Vitamin D receptor (VDR) and genomic actions of 1α,25(OH)$_2$D$_3$

1α,25(OH)$_2$D$_3$ exerts most of its biological effects through the interaction with a member of the superfamily of nuclear receptors, the vitamin D receptor (VDR). Most normal and tumor cells express VDR and respond to 1α,25(OH)$_2$D$_3$ treatment. VDR has a domain structure [286] homologous to that of the other nuclear receptors. Its 427 amino acids encompass a short **N-terminal activation-function 1 (AF-1) domain (A/B)** that contributes to constitutive ligand-independent receptor activation, a **DNA-binding domain (DBD or "C")** containing two zinc-fingers and also playing a role in receptor dimerization, a **flexible "hinge" region (D)** that includes nuclear localization signals and which is thought to confer rotational flexibility between the DBD and LBD and allow for receptor dimerization and interaction with the DNA, and finally the **ligand-binding domain (LBD or "E")**, whose C-terminal end also has ligand-dependent transcriptional activation function (AF-2).

VDR forms heterodimers with members of the same family, mainly the retinoid X receptor (RXR) and possibly also the thyroid hormone receptor (TR) [41,183,324]. VDR and RXR are in constant movement between cytoplasm and nucleus. In absence of 1α,25(OH)$_2$D$_3$, VDR and RXR are able to heterodimerize in the cytoplasm and RXR modulates the importin α-mediated VDR translocation to the nucleus [324]. In the nucleus, the VDR/RXR heterodimer interacts with specific DNA sequences known as vitamin D response elements (VDREs) present in its target genes. Usually, VDREs are direct repetitions of (A/G)G(G/T)TCA hexanucleotide spaced by three or four nucleotides (nt) (DR3 or DR4, respectively) or everted repetitions spaced by six to nine nt (ER6-ER9), and each receptor interacts with a hemisite [113,266,294]. One or more VDRE have been identified in the promoter of 1α,25(OH)$_2$D$_3$ target genes such as CYP24, p21^{CIP1}, osteopontin, osteocalcin, insulin-like growth factor-binding proteins (IGFBP)-1, -3, -5, cyclin C and transient receptor potential cation channel (TRPV) -5,-6 [41].

In the absence of 1α,25(OH)$_2$D$_3$, the VDR/RXR heterodimer is bound to DNA at VDRE and recruits co-repressor proteins as NCoR, SMRT and Alien/TRIP15. The co-repressors have intrinsic histone deacetylase activity (HDAC) or associate themselves with HDAC enzymes promoting chromatin compaction and target genes are silenced. Binding of 1α,25(OH)$_2$D$_3$ to VDR leads to a conformational change of the LBD switching co-repressors for co-activators belonging to p160/SRC family as SRC-1/NCoA-1, SRC-2/NCoA-2/GRIP1/TIF2 and SRC-

3/RAC3. The interaction of co-activators with the AF-2 region of LBD recruits histone acetyltransferases (HAT) and chromatin-remodeling complexes as CBP/p300 and WINAC, respectively, that together mediate chromatin decompaction [273,286]. It has been suggested that co-activators have a temporary interaction and subsequently, they are substituted by the VDR-interacting proteins complex (DRIP/TRAP). This complex recruits the RNA polymerase II and links VDR to the basal transcriptional machinery promoting gene activation. However, the precise order of interaction and action of the different complex is not yet established [286].

Recent studies using microarrays show that about one third of $1\alpha,25(OH)_2D_3$ target genes are repressed [41,227], but how VDR activated by $1\alpha,25(OH)_2D_3$ can repress gene expression is a less characterized mechanism [148,262].

1.3.2 Non-genomic effects of $1\alpha,25(OH)_2D_3$

$1\alpha,25(OH)_2D_3$, like other steroid hormones, can also induce rapid responses (seconds-minutes to hours) independently of gene expression [213]. These non-genomic effects can be mediated by a subpopulation of VDR localized in the cytoplasm or in the plasma membrane, or by another different receptor. Recent studies have localized VDR in the caveolae membrane fraction of different cell lines and associated with actin filaments near the plasma membrane [90,124]. A few studies support that VDR mediates these rapid responses. Several non-genomic actions of $1\alpha,25(OH)_2D_3$ are blocked in $VDR^{-/-}$ mice osteoblasts and also by a mutant VDR lacking the first zinc finger necessary for DNA binding [69,330].

Many non-genomic effects are triggered by $1\alpha,25(OH)_2D_3$ interaction with extranuclear receptors, VDR or others. Thus, opening of voltage-gated Ca^{2+} and Cl^- channels, important for the rapid intestinal calcium absorption or the cytosolic calcium elevation (from the outer medium and/or intracellular sources), the activation or inhibition of different kinases (c-SRC, PKC (α, β, γ), PI3K, JNK, ERK and the activation of phospholipases (PL) C and D and/or phosphatases (PP1 and PP2A) are $1\alpha,25(OH)_2D_3$ non-genomic effects [28,36,147,213,214].

1.3.3 Integration of genomic and non-genomic effects of $1\alpha,25(OH)_2D_3$

Recent results of our laboratory have shown that $1\alpha,25(OH)_2D_3$ activates a non-genomic pathway beginning with the entry of Ca^{2+} from the external medium and continuing with the activation of RhoA GTPase and p38MAPK-MSK kinases. This pathway is necessary for the optimal regulation of $1\alpha,25(OH)_2D_3$ target genes, as well as for the interference with the transcriptional activity of β-catenin/TCF complexes. VDR is required for the activation of this signaling pathway, showing its dual function as nuclear transcription factor and as an extranuclear activator of non-genomic effects [220].

2. Colorectal cancer

Colorectal cancer (CRC) is together with lung, breast and prostate one of the most frequent cancers worldwide [76,138]. CRC is the result of the malignant transformation of epithelial cells lining the inner colon and rectum. CRC has an important geographic component; the incidence is greatest in industrialized countries where consumption of meats, fats and refined carbohydrates *per capita* is high [230]. Other important risk factors beside diet and lifestyle are ethnicity and age. These data suggest an important role of environmental factors in the susceptibility to CRC cancer [111,189,218]. Only a low percentage of CRC (between 5-10%) is attributable to hereditary factors. Thus is the case of two autosomal dominant syndromes characterized for the CRC development at an early age: familial adenomatous polyposis (FAP), caused by the heredity of a mutated copy of adenomatous polyposis coli (APC) tumor suppressor gene [101,149] and hereditary nonpolyposis colorectal cancer (HNPCC), caused by the mutation or epigenetic silencing of some mismatch repair (MMR) genes such as MSH2, MLH1, MSH6, PMS1 or PMS2 [186,194,240].

Most of CRC are sporadic and with a slow development, providing a great opportunity to an early detection. As many as 70% of CRC patients do not show evidence of metastasis. Surgical resection is the therapy of choice for localized tumors (50% of recurrence), but there is no satisfactory treatment when surgery is not curative or for advanced colon cancer [79,191].

2.1 Biology of colonic epithelium

The intestine is the segment of the gastrointestinal (GI) tract extending from the stomach to the anus and, in humans and other mammals, consists of two segments, the small intestine and the large intestine. The large intestine consists of the cecum and colon with the rectum in the final straight portion. The GI tract has a uniform general histology and can be divided into four concentric layers: The **serosa** or peritoneum is the outermost connective tissue layer that covers the abdominal cavity. The ***muscularis externa***, bounded by serosa, consists of an inner circular layer and a longitudinal outer muscular layer; the coordinated contraction of these layers is called peristalsis. The **submucosa** consists of a dense irregular layer of connective tissue with large blood vessels, lymphatics and nerves branching into the mucosa and muscularis. Finally, the **mucosa** is the innermost layer of the GI tract that is surrounding the lumen. In the colon, this layer forms tubules of mucosal epithelium, perpendicular to the luminal surface, so-called Lieberkühn crypts, but lacks of intestinal villi (characteristic of the small intestine) (Figure 2). The mucosa is responsible for the processing and absorption of nutrients as well as for the compaction of the stool [263]. Lieberkühn crypts are lined with short columnar epithelial cells, mostly mucus-secreting

Goblet cells, with interspersed water absorbing **enterocytes** and fewer peptide hormone-secreting **enteroendocrine cells**.

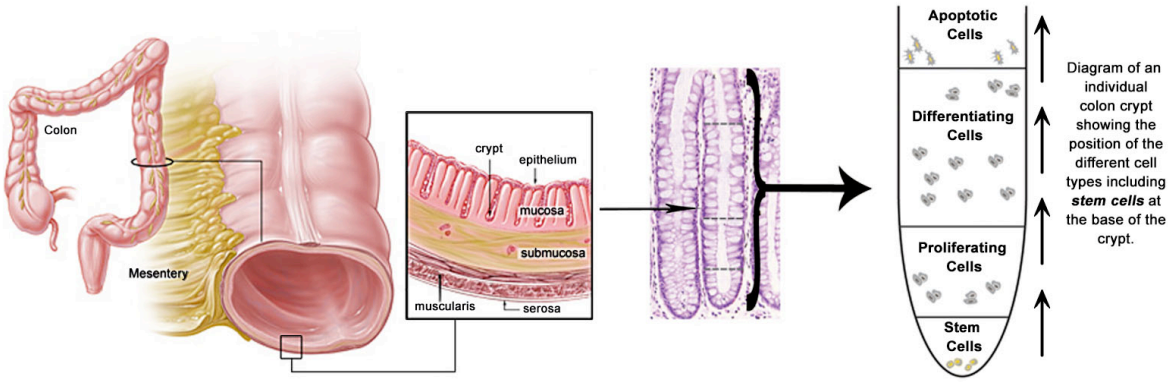

Figure 2. Schematic representation of colonic epithelium. In the colon, the mucosa layer forms the Lieberkühn crypts (tubules of mucosal epithelium, perpendicular to the luminal surface). In the crypt base are located the intestinal stem cells that differentiate into three different mature cell types (goblet cells, enterocytes, and enteroendocrine cells) while they migrate to the intercrypt table at the top of the crypt. The cells of the upper crypt do not have the capacity to regenerate.

The GI epithelium is frequently worn away by the passing food. Therefore, every 4-5 days is renovated [263]. Cells replacement and production in intestinal crypts is achieved by intestinal stem cells (ISCs) located at the Lieberkühn crypt base. These ISCs can produce progeny that undergo a limited number of divisions and ultimately can differentiate, as they migrate to the intercrypt table at the top of the colonic crypt, into the three different mature cell types described previously. When the cells mature, they gradually lose their stemness. Therefore, the cells of the upper crypt do not have the capacity to regenerate [32,187,247,263]. Recently, Lgr5 and Prominin-1 (CD133) have been proposed as colon stem cells markers, allowing a more detailed study of crypts proliferation and differentiation processes [15,337].

2.2 Cause and progression of intestinal neoplasia

In humans, in contrast with mice, most of intestinal cancers affect the colon and not the small intestine. In 1990 Fearon and Vogelstein suggested that CRC is the result of the accumulation of alterations in certain oncogenes and tumor suppressor genes involved in proliferation and cell death control. Although the genetic alterations often occur in a certain order, the total accumulation of changes, rather than their order with respect to one another, is responsible for determining the tumor biological properties. According to the model few changes are sufficient for initiating tumorigenesis [74].

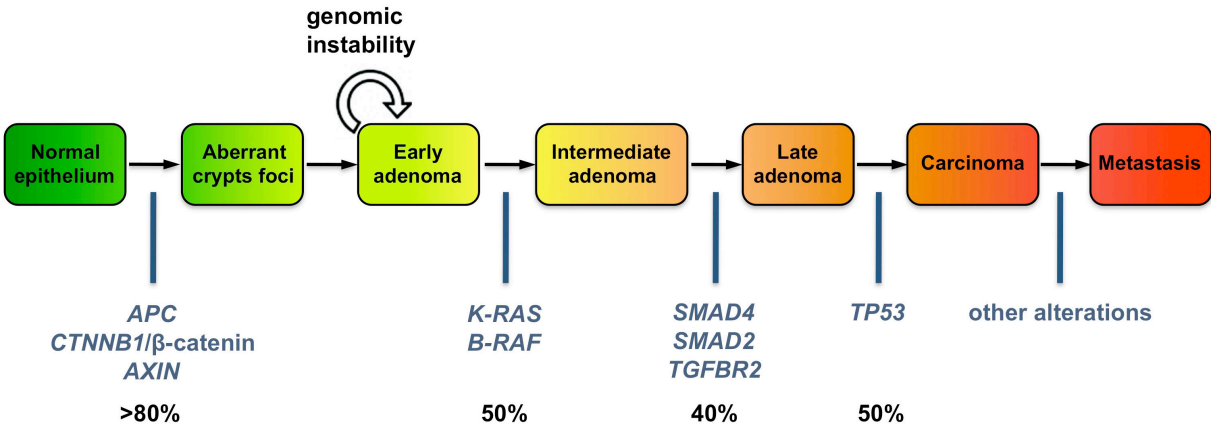

Figure 3. Model of CRC progression. The mutations related with a specific stage and their frequency (percentage of tumors) are shown. The mutator pathway, driven by epigenetic silencing or, less frequently, the mutation of MMR genes is not showed here. Modified from Fearon and Vogelstein 1990 [74].

Aberrant crypt foci (ACF) in colorectal mucosa are proposed to be the earliest morphological lesion in the development of neoplasia. There are two different types of ACF: dysplastic and non-dysplastic [215]. Non-dysplastic ACF are more common, have *K-RAS* mutations and a limited potential to progress to adenoma and ultimately carcinomas. Dysplastic ACF show *APC* mutations and are the precursors of colon cancer [8,288]. The mutation of a gene involved in the canonical Wnt/β-catenin pathway (*APC* or, less commonly, *CTNNB1*/β-catenin or *AXIN*) seems to be sufficient for adenoma initiation, while mutation of *K-RAS* or *B-RAF* and subsequent alterations in the transforming growth factor (TGF)-β pathway (*SMAD4, SMAD2* or *TGFβR2* genes) confer additional malignant features to the adenoma [98,253]. The progression towards carcinoma involves the inactivation of *TP53* gene in 50% of colon tumors [126]. The mechanism involved in the acquisition of invasiveness properties and metastatic capacity is less known. Loss of E-cadherin and ephrin B receptors (EPHB) expression and modification the pattern of claudin 1 expression have been related with these processes [21,57] (Figure 3).

This is known as the suppressor pathway and occurs in 85% of sporadic colon cancer and FAP patients. It is associated to chromosomal instability (CIN) with allelic loss and aneuploidy due to *APC* and *TP53* mutations [80,126,253]. In addition, the epigenetic silencing or less frequently, the mutation of MMR genes drives a second via for CRC, the mutator pathway. These tumors have a normal karyotype but show microsatellite instability (MIN). This pathway causes the HNPCC and 15% of sporadic CRC [129,239].

2.3 Importance of Wnt/β-catenin pathway in CRC

The members of Wnt family are secreted cysteine-rich glycoproteins that participate in multiple developmental events during embryogenesis and have also been implicated in adult tissue homeostasis [179]. In recent years numerous studies have shown that Wnt signaling is very often deregulated in pathological conditions. It is well established that the canonical or Wnt/β-catenin signaling pathway is aberrantly activated in a large number of hereditary and sporadic cases of CRC [100]. In normal epithelial cells, in the absence of Wnt stimulation, cytoplasmic β-catenin levels are normally kept low through continuous proteasome-mediated degradation, which is controlled by a multiprotein complex containing the scaffold protein Axin, the tumor suppressor gene product APC, as well as the casein kinase 1 (CKI), and glycogen synthase kinase 3 (GSK3β), among others. In the nucleus prospective Wnt target genes are in a repressed state [47,197,241] (Figure 4, left panel).

Wnt factors activate responding cells by interacting with the Frizzled (Fz) seven-span transmembrane receptors together with the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6). The binding of Wnt to the receptors leads to activation of the phosphoprotein Dishevelled (Dvl) that possibly recruits Axin or inhibits GSK3β, disrupting the degradation complex and blocking β-catenin degradation. As β-catenin levels rise, it accumulates in the nucleus, where it interacts with DNA-bound T-cell factor (TCF), and lymphoid enhancer-binding protein (LEF) family members to activate the transcription of target genes [47,100,179,197,241] (Figure 4, right panel).

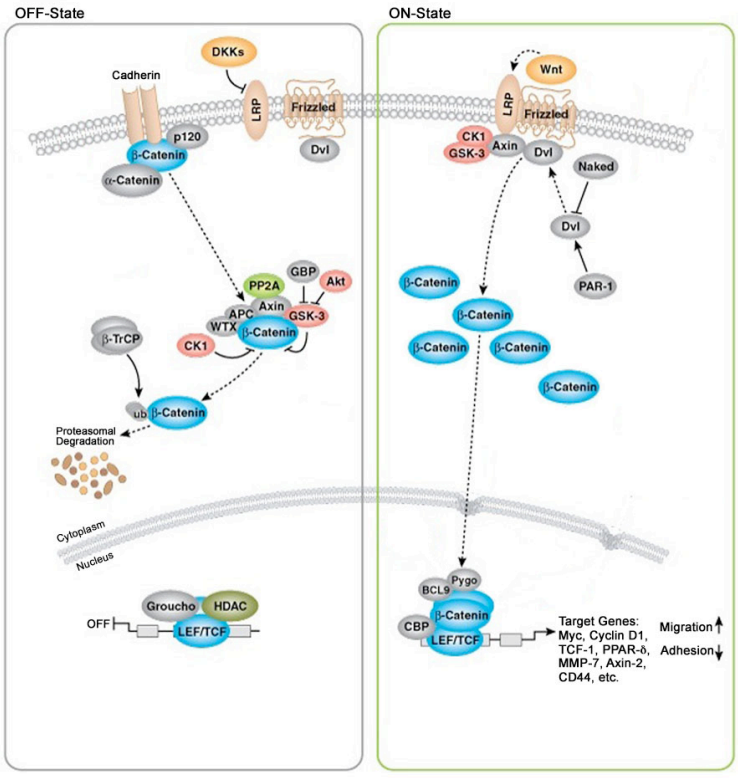

Figure 4. Wnt/β-catenin pathway. In the absence of Wnt stimulation, β-catenin is associated with a multiprotein complex containing APC, axin, CKI and GSK3β proteins. This complex phosphorylate and label β-catenin for proteasome-mediated degradation (left). Wnt factors inhibit the phosphorylation of β-catenin, that accumulates in the nucleus where interacts with TCF/LEF family members and activates the transcription of target genes (right). Modified from Cell signaling technology.

The Wnt/β-catenin signaling pathway has several antagonists that can be divided into two different classes. Both prevent ligand-receptor interactions, but by different mechanisms: members of the first class, which include the secreted Frizzled-related protein (SFRP) family, Wnt inhibitory factor-1 (WIF) and Cerberus, primarily bind to Wnt proteins; the second class comprises certain members of the Dickkopf (Dkk) family, which bind to the LRP subunit of the Wnt receptor complex [144]. In addition, there are several intracellular pathways that interfere Wnt signaling [315].

Homeostasis of the intestinal epithelium is maintained by the balance of cell proliferation, differentiation, and apoptosis. Although many signaling pathways such as BMP, Sonic Hedgehog and Notch are involved in maintaining this equilibrium, Wnt signaling is the main driver of self-renewal in the intestinal epithelium (reviewed in [47,81,252,263,305]).

Wnt signaling is active at the bottom of the crypts [242,303]. In fact, ISCs show nuclear β-catenin and express β-catenin/TCF4 target genes [100,303]. The activity of β-catenin/TCF4 complex is switched off as cells migrate upwards and enter the differentiated cell compartment [303]. Despite many efforts, the location of the source of Wnt ligands has not been elucidated yet. Several Wnt family members are expressed in crypt epithelial cells, thus suggesting paracrine or autocrine signaling in the epithelium [304]. In addition, mesenchymal cells surrounding the crypt bottom have also been proposed as a putative Wnt source [7,125,303].

3. Effects of $1\alpha,25(OH)_2D_3$ in colon cancer

3.1 Vitamin D_3 status and colorectal cancer

Studies using animal models show that a Western-style diet (poor in vitamin D) increases the CRC and other neoplasias risk [204,258,321]. In mutant *APC*^{min} mice, that develop polyps in normal conditions, diets with low vitamin D content increase the number of polyps and diminish the survival in contrast to diets rich in vitamin D [323]. Moreover, in *APC*^{min} mice as well as other animals with chemical carginogen-induced colon tumors, $1\alpha,25(OH)_2D_3$ (or its analogs) treatment reduces the total tumor load [24,72,123,133,145,290].

Ecological and epidemiological studies have demonstrated that solar radiation exposure and $25(OH)D_3$ serum levels are inversely correlated with colon cancer risk [78,86,99,289]. Meta-analysis and cancer prevention trials indicate that vitamin D_3 supplementation to achieve a level of 82 nM of $25(OH)D_3$ can lower the incidence of CRC by 50% [56]. The results of two independent prospective studies with contradictory conclusions have been published [167,311]. However, both studies have deficiencies (low vitamin D_3 doses, limited number of colon cancer cases) that prevent a definitive conclusion. In 2008, the International Agency for Research on Cancer (IARC), from the World Health Organization (WHO), has issued the

Vitamin D and cancer report, where it is recognized that a causal link between vitamin D_3 and colorectal cancer is probable, but more prospective clinical trials and large-scale observational studies are needed.

3.2 Effects of 1α,25(OH)$_2$D$_3$ in colon cancer cells

The anticancer activity of 1α,25(OH)$_2$D$_3$ in colon cancer cells stems mainly from the inhibition of proliferation and the induction of apoptosis and differentiation. The growth-inhibitory action of 1α,25(OH)$_2$D$_3$ has been observed in many human colon cancer cell lines and also in cultured primary human colon adenoma- and carcinoma-derived cells [108,180,225,295,335]. This antimitotic action is mediated by the induction of G_0/G_1 cell-cycle arrest as a result of the up-regulation of the cyclin-dependent kinase (CDK) inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, as well as the repression of transcription factor Id2. Furthermore, 1α,25(OH)$_2$D$_3$ regulates many other genes related to proliferation including *c-MYC, c-FOS* and *c-JUN* and by interfering with certain signaling pathways that control epithelial cell growth (EGF, IGFII) [95,227].

In addition to cell cycle inhibition, 1α,25(OH)$_2$D$_3$ induces apoptosis in colon cancer cells by the regulation of genes that control death pathways. Mainly, 1α,25(OH)$_2$D$_3$ upregulates the pro-apoptotic protein BAK [58,227]. The apoptosis induced by 1α,25(OH)$_2$D$_3$ did not require an intact *TP53* tumor suppressor gene. This would allow the use of 1α,25(OH)$_2$D$_3$ or its analogs for cancer treatment independently of the tumor *TP53* status [58,109].

The antiproliferative action of 1α,25(OH)$_2$D$_3$ and its analogs is commonly linked to stimulation of cell differentiation. 1α,25(OH)$_2$D$_3$ increases the number of intermediate filaments, desmosomes, and microvilli [108,110]. Alkaline phosphatase activity, widely considered as a colon differentiation marker, is also induced by 1α,25(OH)$_2$D$_3$ in primary cultured colon carcinoma cells and in colon cancer cell lines [58,295]. Our group has described the epithelial differentiation promoted by 1α,25(OH)$_2$D$_3$ in SW480-ADH cells, a subpopulation of SW480 colon adenocarcinoma cells that express VDR [225]. This differentiation is associated with the induction of E-cadherin expression, the main component of *adherens junctions*. In addition, 1α,25(OH)$_2$D$_3$ induces the expression of the components of tight junctions occludin, *Zonula occludens* (ZO)-1, ZO-2 and vinculin [225]. Moreover, 1α,25(OH)$_2$D$_3$ inhibits the Wnt/β-catenin pathway (see section 2.3) and represses its target genes, contributing to the maintenance of a differentiated phenotype and the inhibition of cell cycle [225]. The effects of 1α,25(OH)$_2$D$_3$ over E-cadherin and β-catenin expression have also been observed in other colon cancer cell lines as HT-29, CaCo2, SW1417 and LS174T [225].

4. Cell adhesion in epithelial tissue

4.1 *Adherens junctions*

Together with tight junctions and desmosomes, *adherens junctions* are the molecular complexes responsible of cell-cell adhesion. *Adherens junctions* play a key role in initiating and stabilizing cell-cell contacts. In addition, they enhance the ability of the cell to sense and respond to its environment, for instance, by bringing neighbor cells together and therefore allowing activation of membrane receptors, which bind ligand tethered-ligands. *Adherens junctions* are dynamic structures with very precise and fast regulation. Their main components are: cadherins, β-catenin, p120-catenin and actin cytoskeleton structures [104] (Figure 5).

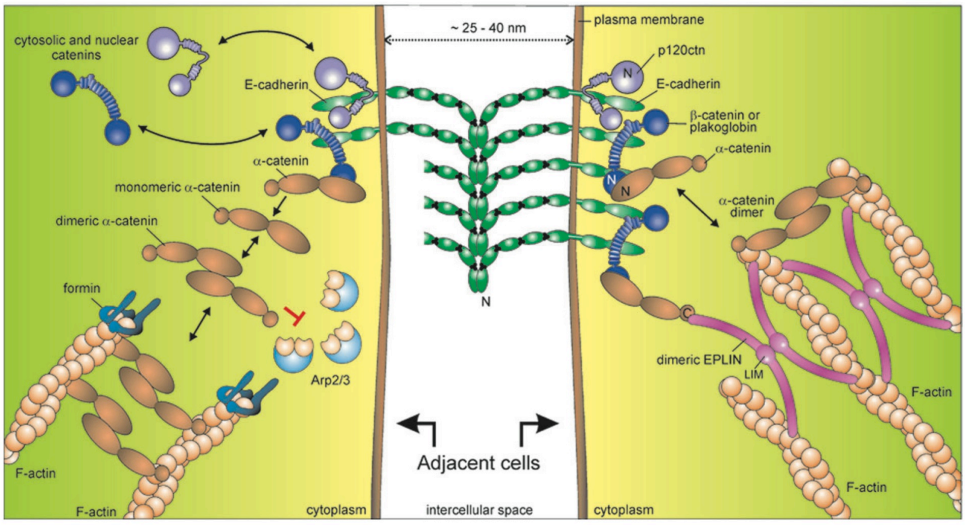

Figure 5. *Adherens junctions*. Schematic representation of E-cadherin interactions. Through its ectodomain E-cadherin establishes homotypic interactions with E-cadherin molecules of neighboring cells in a calcium-dependent manner. The intracellular domain of E-cadherin interacts with α-, β-, and p120-catenin that stabilize E-cadherin in the membrane and connect it to the actin cytoskeleton. From van Roy and Berx 2008 [307].

4.1.1 E-cadherin

E-cadherin (encoded by *CDH1* gene) is primarily expressed in epithelial cells and is the core transmembrane protein of the *adherens junctions*. Its ectodomain establishes early cell-cell contacts through individual and relatively weak homotypic interactions with E-cadherin molecules of neighboring cells in a calcium-dependent manner. This initial event typically takes place at the tip of filopodial or lamellipodial projections termed *puncta*. The sequential binding of several catenins to E-cadherin cytoplasmic tail stabilizes E-cadherin in the membrane and connects it to the actin cytoskeleton and to other signaling molecules (Figure

5). These events allow contact strengthening by formation of cadherin clusters through the assembly of adjacent *puncta* that spread laterally generating zipper-like structures, which finally seal the membranes (reviewed in [112]). In many epithelial-derived tumors, loss of E-cadherin correlates with an increase in tumor cell invasiveness and motility, a process known as epithelial-mesenchymal transition, which points to a tumor suppressor role for E-cadherin (reviewed in [291]).

4.1.2 p120-catenin

p120-catenin (p120ctn) is a key regulator of *adherens junctions* stability. p120ctn binding to E-cadherin in its juxtamembrane domain prevents cadherin internalization and degradation. Conversely, p120ctn dissociation from E-cadherin promotes cadherin recycling or degradation by inducing vesicle formation. Some molecules have been proposed as positive (afadin, Rap1) or negative (Hakai, presenilin-1) regulators of the p120ctn-E-cadherin interaction [320].

Decrease or even complete silencing of p120ctn expression, accompanied by a downregulation of E-cadherin levels, is found in several human tumors (i.e. colon, prostate, breast and lung) [6]. Paradoxically, established tumor cell lines rarely present p120ctn downregulation [53].

4.2 Tight junctions

The epithelial tight junction, or *zonula occludens* (ZO), seals cells together at a subapical location and functionally separates the plasma membrane into an apical and a basolateral domain and serves as a selectively permeable barrier to regulate paracellular diffusion. This junction is one of the most characteristic structural markers of the polarized epithelial cell. Some components of tight junction are structural proteins as ZO-1 and transmembrane proteins such as claudins and occludins [73,94,193].

4.2.1 Occludin

Occludin is a four-pass integral plasma-membrane protein located specifically at tight junctions. Numerous studies have indicated that occludin plays an important role in the regulation of tight junction integrity. However, recent studies indicated that tight junctions are formed in the absence of occludin and questioned the role of occludin in their assembly [254]. The cytoplasmic domain of occludin may be involved in the regulation of tight junctions through intracellular signaling. Occludin directly interacts with c-Src, ERK1/2, PP2A, and PP1 [19,20,261]. Therefore, occludin is likely to play a regulatory role in tight junction integrity.

5. Epithelial-mesenchymal transition

An epithelial-mesenchymal transition (EMT) is a biological process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype. This includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of extracellular matrix (ECM) components [141]. The completion of an EMT is signaled by the degradation of underlying basement membrane and the formation of a mesenchymal cell that can migrate away from the epithelial layer in which it originated. The phenotypic plasticity afforded by an EMT is revealed by the occurrence of the reverse process, a mesenchymal-epithelial transition (MET), which involves the conversion of mesenchymal cells to epithelial derivatives. Relatively little is known about this process (reviewed in [142]).

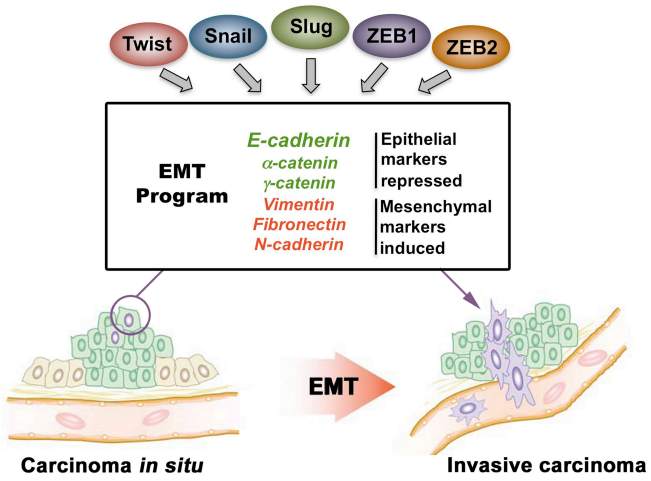

Figure 6. Drivers and mediators of EMT. In early stage tumor cells (green) the accidental overexpression of master regulators of EMT, such as Twist, Snail, Slug, ZEB1 and ZEB2, leads to dramatic changes in gene expression profile and cellular behavior. These EMT-related proteins repress the expression of *E-cadherin* via E boxes in its promoter and trigger expression of an entire EMT transcriptional program. Modified from Kang and Massagué 2004 [143].

A hallmark of EMT is the loss of E-cadherin expression. This loss increases tumor cell invasiveness *in vitro* and contributes to the transition of adenoma to carcinoma in animal models [291]. Several developmentally important genes that induce EMT have been shown to act as E-cadherin repressors. Thus, Snail (*SNAI1* and *SNAI2*), zinc-finger E-box binding homeobox (ZEB: *ZEB1* and *ZEB2*) and basic helix-loop-helix (bHLH: *E47*, *TWIST* and *E2-2*) families mediate the transcriptional repression of E-cadherin ([274] and references therein). The expression patterns of Snail, ZEB and bHLH factors in different human carcinomas, together with functional studies, indicate that the various factors have different roles during tumor progression, with a more prominent role for *SNAI1* in the induction of EMT in primary tumors, whereas the other factors are involved in maintaining the migratory phenotype [233,234] (Figure 6).

5.1 Snail family factors

SNAI1 (Snail) and *SNAI2* (Slug), members of the Snail superfamily, are zinc-finger transcription factors that share a common organization with a central region that is highly divergent between them. In *Drosophila* these proteins mediate the development of the mesoderm, neuroectoderm and the other organs [16]. As a suppressor of E-cadherin expression, Snail binds to E-boxes (CANNTG) present in the *CDH1* gene promoter, and represses its transcription [22,39]. In addition, induction of Snail expression triggers a complete EMT [39,103].

An inverse correlation between E-cadherin and Snail expression has been described in cultured lines established from oral squamous cell carcinoma, melanoma, pancreatic and hepatocellular carcinoma [122,139,244,325]. Moreover, previous studies of our laboratory showed that *SNAI1* expression was associated with the downregulation of VDR in CRC, with therapeutic implications for vitamin D treatment of Snail-negative tumors [169]. More recently, our group also showed that *SNAI2* is able to repress VDR alone or in cooperation with *SNAI1* [168].

5.2 ZEB family factors

The ZEB family of transcription factors contains two members, ZEB1 and ZEB2. They are characterized by the presence of two zinc-finger clusters at each end and a central homeodomain. ZEB factors are expressed during development in the central nervous system, heart, skeletal muscle and haematopoietic cells [233]. These transcription factors repress E-cadherin by binding to paired E-box sites in the proximal promoter. Both factors have been shown to induce EMT, and to participate in TGFβ signaling through binding to Smad co-activators, although the links between these two processes had not been clearly defined [209,245,246].

5.3 bHLH family factors

The bHLH proteins have a common structure with two parallel amphipatic α-helices joined by a loop, required for dimerization, and one helix containing basic amino-acid residues that facilitate DNA binding. The bHLH proteins can be classified into discrete categories. TWIST1 is a member of class I (also known as E-proteins) bHLH proteins. It has been implicated in cell lineage determination and differentiation. TWIST1 is overexpressed in various human solid tumors including numerous types of carcinomas as well as sarcomas, gliomas, neuroblastomas, and melanomas [64,68,118,164,216,322,334]. Moreover, exogenous overexpression

of TWIST1 increases the invasive and metastatic abilities of human cancer cells by promoting the downregulation of E-cadherin and the induction of EMT [164,192,322].

6. Cystein cathepsins

Degradation of proteins is brought about by proteolytic enzymes, proteases, which based on their catalytic mechanisms can be sorted into five major classes; the serine-, cysteine-, aspartic-, threonine-, and metallo-proteases.

Cathepsins are lysosomal peptidases, which belong mainly to the cysteine, but also to serine or aspartic protease classes. The **human cysteine cathepsin** family comprises 11 members (cathepsins B, C, H, F, K, L, O, S, V, W X/Z). Some of them are ubiquitously expressed, such as cathepsins B, L, H and C, whereas several newly found family members as cathepsins K, W, and X are expressed in specific cells and tissues [35,159,317,319]. The most important human cysteine cathepsins are cathepsins B, H, L, and S, which have common ancestors and are related to papain.

Cathepsins participate in general protein turnover and can also perform specific functions in neovascularization [200], cell growth, antigen presentation, bone remodeling, and tissue homeostasis (reviewed in [296,300]). Interestingly, cathepsins also have a role outside the lysosomes. When cathepsins are secreted into the extracellular space, they participate in degradation of the ECM or induction of fibroblast invasive growth, and when they are released into the cytosol they may execute a programmed cell death [75,153,296].

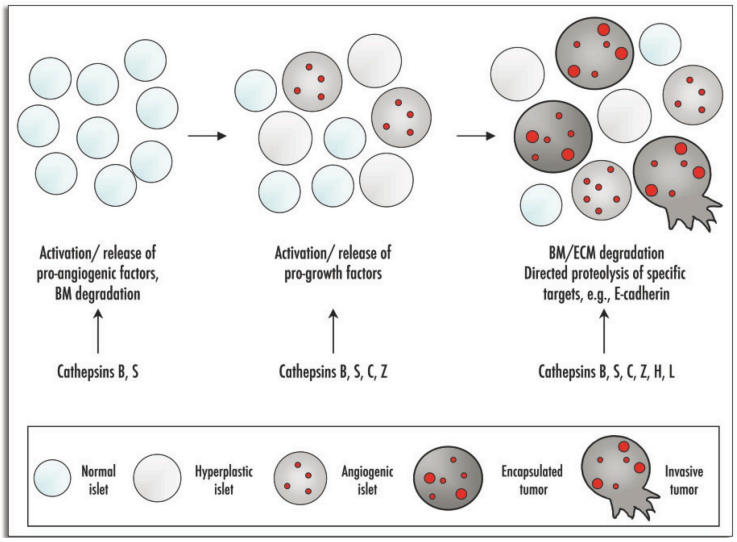

Figure 7. Multiple roles for cysteine cathepsins during tumor progression. The RIP1-Tag2 model* of islet cell carcinogenesis is used to illustrate that cysteine cathepsins are collectively important for tumor angiogenesis, cell proliferation, tumor growth, and tumor invasion. Specific cathepsin are shown in each case. From Joyce and Hanahan 2004[140].

* For explanation see page 85.

Cysteine cathepsins have been implicated in multiple physiological disorders and also in tumor progression, at early steps of tumor invasion and angiogenesis, and at late steps of metastasis and drug resistance [140] (Figure 7).

7. Cystatins: protein inhibitors of cysteine cathepsins

Cystatins are protein inhibitors of cysteine proteases of the papain family [17,297,298]. They constitute a powerful regulatory system for endogenous cysteine proteinases, which are often secreted or leaking from the lysosomes of dying cells or diseased cells. Cystatins inactivate target proteases reversible and competitively by indirect blockage of the catalytic center of these enzymes, thereby preventing substrate docking and cleavage. They form very stable bimolecular complexes with the proteases, keeping them inactive for hours or weeks. Cystatins are widely distributed in animals, plants and protozoa, both intracellularly and extracellularly, which indicates an important physiological role [17,132,298].

Cystatins are all related by structure and function to an inhibitor of cysteine proteinases that was first described in egg white and called as chicken egg white (CEW) cystatin. Cystatins have been evolutionary related forming the **Cystatin Superfamily*** . The members of the superfamily were grouped into three families or "types" on the basis of their location, size and complexity of polypeptide chains [18,255]. **Type 1 cystatins** are found primarily intracellularly, contain about 100 amino-acid residues (~ 11 KDa) and lack disulfide bonds. There are two human representatives, cystatin A and B. The type 1 cystatins have been called "stefins" to stress their difference from other cystatin superfamily members. **Type 2 cystatins** are mainly extracellular secreted proteins, contain about 120 amino-acid residues (~ 14 KDa) and two intrachain disulfide bonds. The salivary cystatins (**cystatin D** and others), cystatin C, E/M and CEW cystatin belong to this family. **Type 3 cystatins** comprise the plasma kininogens and may therefore also be called the kininogen family. They are multi-domain proteins with high molecular weight (~60-120 KDa). They contain additional disulfide bonds and are also glycosylated. There are three types of kininogens, the low molecular weight kininogens (LMWK), and high molecular weight (HMWK), and T-kininogens, only found in rats.

* In the recently developed MEROPS database (http://merops.sanger.ac.uk) proteases and their inhibitors are classified into clans and families according to their evolutionary relationship. Proteases and/or inhibitors with statically significant similarities in amino-acid sequence are grouped into families. Clans are formed by related families. The cystatins entered into the classification schemes to date all belong to MEROPS Family I25 of Clan IH.

7.1 Type 2 cystatins

Type 2 cystatins are synthesized as preproteins with a signal peptide and, as mentioned previously, the members of this family are mainly extracellular, secreted proteins, although some have also been found intracellularly [1,2,105,202]. The family includes mammalian cystatins: cystatin C of several different species [70,102,117], **cystatin D** [82,83], cystatin E/M [206,279,331], cystatin F/leukocystatin [105,198,207], cystatins S, SA and SN [134-136], and also CEW cystatin [299].

The best studied representatives of type 2 cystatins are CEW cystatin and human cystatin C, the latter being the most abundant of the inhibitors of the cystatin superfamily in all human body fluids examined, with the highest concentration in seminal plasma and cerebrospinal fluid [1,2,217]. Recently, cystatin C has been proposed as a useful indicator of glomerular filtration rate (reviewed in [170]). In addition, cystatin C has been proposed as a TGFβ receptor antagonist, playing important roles in tumor progression[275,276].

Cystatin E/M has only ~ 30% sequence identity with other type 2 cystatins and, unlike all other human type 2 cystatin genes, it is not located on chromosome 20, but on chromosome 11 [281]. Cystatin E/M is quite ubiquitous and is expressed in a variety of normal human tissues [206,279]. Cystatin E/M has been widely studied because of its role as tumor suppressor mainly in breast cancer [150,270,333].

7.2 Cystatin D

Cystatin D was first identified as the product of a gene located in the cystatin multigene locus on chromosome 20 [84] with a high degree of homology to the cystatin C gene. It contains all the classical features of a type 2 cystatin (Figure 8).

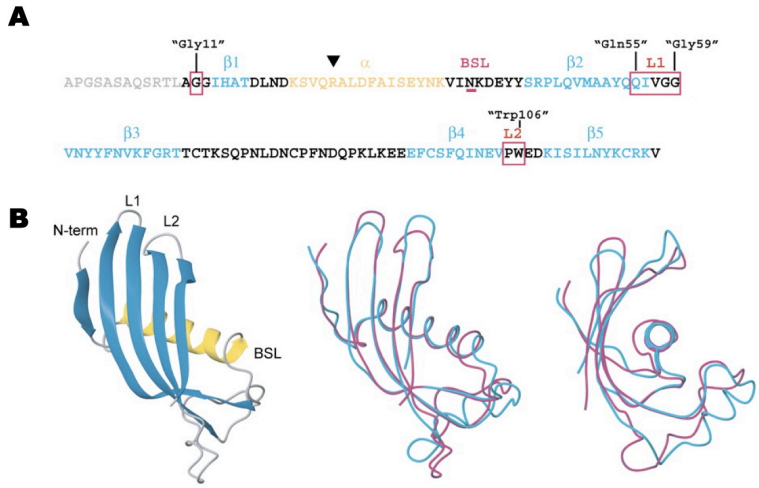

Figure 8. Cystatin D structure and comparison with CEW cystatin. (A) Amino-acid sequence of the cystatin D form crystallized, recombinant human cystatin D. The secondary structure elements are indicated in yellow for α-helix and blue for β-sheet. The motifs known to be important for the inhibition of papain-like enzymes by other cystatins are marked by red boxes, and some well-conserved residues in them are indicated according to human cystatin C numbering. The putative legumain-binding site (BSL) is also indicated, and the presence of an Asn residue in this loop is pointed out (underlined). The position of the residue corresponding to a gene polymorphism (Cys/Arg) is marked by an arrowhead. **(B)** Ribbon representation of the cryo structure of human cystatin D viewed from the front (left) and representations of the aligned structures of human cystatin D (in magenta) and CEW cystatin (in cyan) are viewed from the front (middle) and from the C-terminal end of the α-helix (right). Modified from Alvarez-Fernandez *et al.* 2005 [5].

Cystatin D is produced as a preprotein of 142 amino-acid residues, the first 20 residues constitute a typical signal peptide [5,82]. Its complete amino-acid sequence displays 55% identical residues compared with the cystatin C sequence, with well conserved sequence motifs known to be essential for cysteine peptidase inhibition [82] (Figure 9).

However, cystatin D has a narrower inhibitory profile than other cystatins: it inhibits cathepsin S, H and L, but not cathepsin B [12,82]. Moreover, cystatin D also has a more restricted pattern of tissue expression and is only found in parotid and submandibular glands as well as in saliva, and in small amounts in tears, but could not be detected in seminal or blood plasma, milk or cerebrospinal fluid [83]. Two alleles of the cystatin D gene are known, coding for either Cys or Arg as residue 26 [11]. It has been shown that this variation has no effect on the enzyme-binding properties of the inhibitor [12].

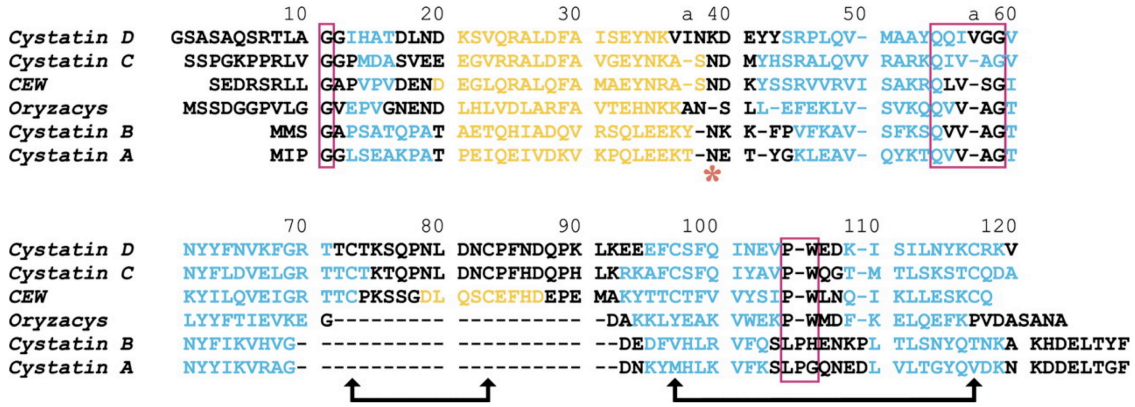

Figure 9. Alignment of several members of cystatin superfamily. Sequence alignment of cystatin D and known type 1 and 2 cystatins (human cystatin A, human cystatin B, CEW cystatin, human cystatin C from the dimer structure, and the plant cystatin, oryzacystatin). The conserved papain-binding site is marked by boxes in magenta. The red asterisk indicates the position of the Asn residue, which is necessary for legumain inhibition. Arrows indicate the two conserved disulfide bridges in type 2 cystatins. From Alvarez-Fernandez *et al.* 2005 [5].

Even though cystatin D was already described in 1991 [82] little is know about its biology or function besides it may play a protective role against proteinases present in the oral cavity.

OBJECTIVES

$1\alpha,25(OH)_2D_3$ is one of the most important regulators of gene expression in higher organisms, and numerous evidences support a preventive and perhaps therapeutic effect of $1\alpha,25(OH)_2D_3$ in colon cancer.

Previous transcriptomic studies of our laboratory showed that $1\alpha,25(OH)_2D_3$ changes drastically the gene expression profile in human colon cancer cells. The level of *CST5* RNA in SW480-ADH cells increased following $1\alpha,25(OH)_2D_3$ treatment.

The aim of this Thesis has been to analyze the relation between $1\alpha,25(OH)_2D_3$ and cystatin D and their biological activity in human colon cancer.

The main goals have been:

1. To validate and analyze the mechanism of the $1\alpha,25(OH)_2D_3$-mediated regulation of cystatin D expression, as well as the role of this protease inhibitor in the effect of $1\alpha,25(OH)_2D_3$ on human colon cancer cells.

2. To characterize the putative antitumor actions of cystatin D in colon cancer.

3. To study cystatin D expression in human colon tumors.

4. To examine the gene expression profile induced by cystatin D in human colon cancer cells.

MATERIALS and METHODS

1. Cell Culture

Several human colon cancer cell lines including SW480-ADH, SW480-R, HCT116, LS174T, Colo205, and CaCo2, as well as the embryonic human kidney cell line HEK-293T were used. The sublines SW480-ADH and SW480-R, provided by Drs. F. X. Real (CNIO, Madrid) and Antonio García de Herreros (Institut Municipal d´ Investigació Mèdica, Barcelona), were obtained from SW480 cells by limit dilution. These cells maintain, in a stable way, their different morphology and response to $1\alpha,25(OH)_2D_3$. Cells were maintained in RPMI medium supplemented with 10% FBS (Invitrogen), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Treatment with $1\alpha,25(OH)_2D_3$ were performed in RPMI supplemented with charcoal-treated serum to remove liposoluble hormones. $1\alpha,25(OH)_2D_3$ was provided by Drs. R. Boullion and M. Verstuyf (Leuven University, Belgium). As control, in each experiment the equivalent volume of isopropanol (vehicle) was used. Unless another specific mention, cells were treated with 100 nM of $1\alpha,25(OH)_2D_3$ or vehicle for the times indicated in each case. In certain experiments, cells were treated with 9-*cis* retinoic acid, *all*-trans retinoic acid or docosahexaenoic acid (all from Sigma), and ethanol was then used as vehicle. Actinomycin D (2 µg/ml in ethanol, Sigma), an inhibitor of transcription by RNA polII, or cycloheximide (8 µg/ml in DMSO, Sigma) an inhibitor of protein synthesis were used in certain experiments. Phase-contrast images of culture cells were captured with a Leica DC300 digital camera mounted on an inverted Leitz Labovert FS Microscope. All the images were processed using Adobe Photoshop CS2 software.

2. Normal and tumor tissue samples from CRC patients

Normal and tumor tissue samples from 32 patients with colon cancer were analyzed by western blot. All patients gave written informed consent and the protocol was approved by the Research Ethics Board of the Hospital Universitario Puerta de Hierro, Madrid. Samples were obtained immediately after surgery, immersed in RNA later (Ambion-Applera), snap-frozen in liquid nitrogen and stored at -80ºC until processing. Tumors were considered sporadic since they do not show familiar or clinical history of FAP or HNPCC. These tumors were examined by two different pathologist for: (a) confirm adenocarcinoma diagnostic and presence of at least 75% of tumor tissue in the sample, (b) establish the histological level of the tumor, and (c) verify the absence of tumor cells in normal tissue.

3. Antibodies

The different primary and secondary antibodies used in the experiments, as well as their origin and concentrations are shown in tables below.

Table I. Primary antibodies

Antigen	Species		Application		References
	Monoclonal	Polyclonal	WB	IF	
c-MYC	Mouse		1/250		Santa Cruz Biotechnology
Cyclophylin A		Rabbit	1/1000		Upstate
Cystatin C		Goat	1/1000		R&D Systems
Cystatin D		Rabbit	1/200	1/100	Prof. C. López-Otín
Cystatin SN	Mouse		1/250		R&D Systems
E-cadherin	Mouse		1/2000	1/100	BD Transduction Laboratories
Lamin B	Mouse		1/2000		Santa Cruz Biotechnology
Occludin		Rabbit	1/2000	1/100	Zymed
p120-catenin	Mouse		1/1000	1/100	Prof. A. Reynolds
Snail1		Rat	1/20		Dr. K-F. Becker
VDR	Rat		1/1000		Chemicon
β-actin		Goat	1/2000		Sigma
β-catenin	Mouse		1/1000	1/100	BD Transduction Lab.

WB: Western blot, IF: immunofluorescence

Table II. Secondary antibodies

Name, antigen	Dilution	Company
HRP-conjugated anti-rabbit IgG	1/10000	MP Biomedicals
HRP-conjugated anti- mouse IgG	1/10000	Promega
HRP-conjugated anti-goat IgG	1/5000	Santa Cruz Biotechnology
HRP-conjugated anti-rat IgG	1/5000	Santa Cruz Biotechnology
Alexa Fluor 488, goat anti-rabbit	1/250	Molecular Probes
Alexa Fluor 488, donkey anti-mouse	1/100	Molecular Probes

HRP: horseradish peroxidase

4. Oligonucleotides

4.1 Oligonucleotides for *CST5* promoter cloning

Table III.

Primers	Sequence	AT
-1867/+262	F: ACGCGTCCGCAGGATCACCTTCAG R: AGATCTTGTACTCGCTGATGGCAAAG	63ºC
-1128/+262	F: ACGCGTCACAGGTGTGGACAAAGTGG R: AGATCTTGTACTCGCTGATGGCAAAG	63ºC
-650/+262	F: ACGCGTTCCAGGAGCTTCCTCTTCCT R: AGATCTTGTACTCGCTGATGGCAAAG	62ºC
-251/+262	F: ACGCGTGAATCCAGAGTGAGCCAAGC R: AGATCTTGTACTCGCTGATGGCAAAG	62ºC

AT: annealing temperature

4.2 Oligonucleotides for cystatin D mutants

Table IV.

Primers	Sequence	AT
W108G	F: CCAGATCAATGAAGTTCCCGGGGGAGGATAAAATTTCCATTC R: GAATGGAAATTTTATCCTCCCCGGGAACTTCATTGATCTGG	55ºC
Δ1-12	F: CCTTGATGGTGGCCGTGGCCGGCATCCATGCCACAGACCCTC R: GAGGTCTGTGGCATGGATGCCGGCCACGGCCACCATCAAGG	55ºC

AT: annealing temperature

4.3 Oligonucleotides for chromatin immunoprecipitation assay

Table V.

Primers	Sequence	AT
CST5 -840/-571	F: CCACAGTGACGCTTGGTCTA R: GTCTGGGCAATAGAGCCGTA	60ºC
CST5 (negative control)	F: ATCTCCCAGAGAGCAAAGCA R: GAATCCAGAGTGAGCCAAGC	58ºC
CYP24 -665/-380	F: CGTTTCCTCCTGTCCCTCTC R: TGCCTTCCTGGGGGTTATCTC	60ºC

AT: annealing temperature

4.4 Oligonucleotides for RT-PCR

Table VI.

Primers	Sequence	AT
TBP	F: TCTGGGATTGTACCGCAGC R: CGAAGTGCAATGGTCTTTAGG	59ºC
SDHA	F: TGGGAACAAGAGGGCATCTG R: CCACCACTGCATCAAATTCATG	59ºC
UBC	F: ATTTGGGTCGCGGTTCTTG R: TGCCTTGACATTCTCGATGGT	59ºC
CDH1	F: AGAACGCATTGCCACATACACTC R: CATTCTGATCGGTTACCGTGATC	60ºC
SNAI1	F: CACTATGCCGCGCTCTTTC R: GGTCGTAGGGCTGCTGGAA	68ºC
SLUG/SNAI2	F: GGCAAGGCGGTTTTCCAGAC R: GCTCTGTTGCAGTGAGGGC	59ºC
TWIST	F: CATGTCCGCGTCCCACTAG R: TGTCCATTTTCTCCTTCTCTGG	59ºC
ZEB1	F: GCCAATAAGCAAACGATTCTG R: TTTGGCTGGATCACTTTCAAG	55ºC
ZEB2	F: TATGGCCTACACCTACCCAAC R: AGGCCTGACATGTAGTCTTGTG	59ºC
CST5	F: CCTCTGCAGGTGATGGCTG R: GGACTTGGTGCATGTGGTTC	61ºC
LEF1	F: CGAAGAGGAAGGCGATTTAG R: GTCTGGCCACCTCGTGTC	60ºC

AT: anealing temperature

5. Plasmids

For transactivation assays two different types of constructs were used: reporter plasmids and expression vectors. The reporter plasmids have the cDNA of a reporter gen controlled by the promoter of interest. Those used in this work have the *Firefly* luciferase reporter gene under the control of the following DNA fragments: four copies of VDRE upstream of a minimal *herpes simplex* timidine kinase (*tk*) promoter (4xVDRE-tk-Luc, provided by Dr. C. Carlberg,

University of Kuopio, Finland); three copies of wild type (TOP-flash) or mutated (FOP-flash) TCF/LEF-1 binding sites upstream of a minimal c-*fos* promoter (a gift from Dr. H. Clevers, Utrecht, The Netherlands); four copies of wild-type (4xCBF1wt-Luc) or mutated (4xCBFmut-Luc) CBF1 binding sites upstream of a luciferase gene (provided by Dr. Diane Hayward, John Hopkin´s University, Maryland, USA); the -987/+92 human *CDH1* promoter fragment (provided by Dr. A. García de Herreros, Institut Municipal d'Investigació Mèdica, Barcelona); the -266/+352 and -96/+352 human *c-MYC* promoter fragments (P1P2myc-Luc and P2myc-Luc, respectively, provided by J. León, Universidad de Cantabria, Santander); the -1876/+262, -1128/+262, -650/+262, -251/+262 fragments of *CST5* promoter (described in section 7). As internal control of transfection efficiency, pRLTK (Promega) containing a *Renilla reniformes* (RLuc) reporter gene controlled by *tk* promoter was used.

For ectopic protein expression, we used expression vectors in which a strong promoter drives the transcription of the cDNA of interest. The expression vectors used were: human VDR (provided by Dr. M. Zenke, University of Aachen, Germany) or a truncated VDR lacking the eleven carboxy-terminal amino-acids (ΔAF2, donated by Prof. Ana Aranda from our Institute), both in pSG5, and cystatin D in pcDNA3.1 (described in section 7). Three different cystatin D mutants, with reduced antiproteolytic activity, all in pcDNA3.1, were also used (described in section 7).

6. *In silico* analysis of the human *CST5* gene promoter

The sequence of the human *CST5* gene was obtained from EMSEMBL database. The presence of putative VDR binding sites in a sequence of 2 Kb before the transcription start site and the first exon was examined. The effect of variation of every single nucleotide [314] in comparison with the consensus DNA binding motif, sequence RGKTCA (R = A/G, K = G/T and D = A/G/T), was considered. DR3 and DR4 consensus sites or those with one different nucleotide from consensus were looked up. Moreover, the conservation of these putative sites in other species was carried out using the USCS genome Browser database (in *Macaca mulatta* genome).

7. DNA cloning and mutagenesis

Several fragments of the *CST5* promoter (-1867/+262, -1128/+262, -650/+262, -251/+262) were amplified by PCR using genomic DNA from SW480-ADH cells as template and the high-fidelity *Pfx* DNA polymerase (Invitrogen). The forward and reverse primers used are described in section 4.1. The resulting products were cloned into the pCRII-TOPO vector

(Invitrogen) and sequenced. Subsequently, they were subcloned as *MluI/BglII* fragments into the promotorless pGL3basic *Firefly* luciferase (Luc) expression vector (Promega).

pcDNA3.1-CST5 plasmid was obtained by subcloning the full-length human CST5 cDNA from the pEMBL19 plasmid (provided by Prof. Carlos López Otín, Universidad de Oviedo, Asturias) by digestion with *EcoRI* and *XbaI*.

Directed mutagenesis of CST5 cDNA was carried out using Quick Change® Site Directed Mutagenesis kit (Stratagene) according to the manufacturer's guidelines. For this purpose, forward and reverse primers (described in section 4.2) were designed containing the desired point mutation (W108G) or deletion (Δ1-12) in the middle of the primer sequence. The primers were extended by PCR using the high-fidelity *Pfu* DNA polymerase (Promega). The mutations consisted in changing the *Trp* (TGG) in position 108 for a *Gly* (GGG) in the point mutation, and the deletion of the first 12 amino acids (shown in Figure 34). For the single CST5 W108G mutant the pcDNA3.1-CST5 was used as template. For the double CST5 W108G/Δ1-12 mutant the pcDNA3.1-CST5 W108G mutant was used as template. The PCR reactions were digested with *DpnI* restriction enzyme (Stratagene) to digest the parental DNA. Plasmids were subjected to DNA sequencing to verify the mutation and fidelity of the whole cDNA sequence.

8. Luciferase expression analysis

For transient transactivation assays, cells cultured in 24-well dishes were transfected in triplicate using the jetPEI™ transfection reagent (PolyPlus Transfection). Transactivation assays contained 20 ng of RLuc expression vector pRLTK, 200 ng of Luc promoter reporter and the indicated quantities of expression vectors in each case. The amount of total DNA transfected in every point of each experiment was made equal with pcDNA3 (Invitrogen). If necessary, 24 h post-transfection the cells were changed into a charcoal-treated serum-containing medium and treated with $1\alpha,25(OH)_2D_3$ or vehicle. After 48 h of transfection or treatment with the hormone, depending on the experiment, Luc and RLuc activities were measured using Dual Luciferase™ Reporter Assay System and a Glomax 96-microplate Reader luminometer (both from Promega). Luc activity was normalized to the RLuc activity (Luc/RLuc). Mean values and standard deviations of triplicates were calculated. All the experiments were performed at least three times.

9. Generation of cells stably expressing wild type or mutant CST5

SW480-ADH, LS174T and HCT116 cells stably expressing cystatin D were generated by transfection with pcDNA3.1-CST5, pcDNA3.1-CST5 W108G, pcDNA3.1-CST5 W108G/Δ1-12 or pcDNA3.1 plasmids (control, mock), using jetPEI™ transfection reagent (PolyPlus Transfection), followed by selection with 0.5 mg/ml (SW480-ADH and LS174T) or 2 mg/ml (HCT116) G418 (Sigma) for two or four weeks. Each single antibiotic resistant clone was isolated using cloning cylinders. Cystatin D expression was verified by western blot.

10. Gene silencing

To knock-down *CST5* expression SW480-ADH cells were seeded in 35 mm dishes and infected with lentiviral particles containing a U6 promoter driving a short hairpin RNA (shRNA) targeting the respective RNA. Mission® TRC shRNA (Sigma) lentiviral particles against human *CST5* or scramble negative control were used. After infection the cells were treated with 1 μg/μl puromycin (Sigma). The transfection efficiency was estimated using in parallel lentiviral particles codifying the TurboGFP gene (clone SHC003; Sigma). Control cells were infected with lentivirus bearing a non-targeting shRNA that activates the RISC complex and the RNA interference (RNAi) pathway but that contains at least five mismatched nucleotides compared with any human gene (clone SHC002; Sigma).

11. Total RNA extraction and quantitative RT-PCR

Total RNA was isolated with the RNeasy Kit (Qiagen). The extracted RNA was analyzed electroforetically using 1% agarose gels and stained with SYBR® Safe DNA gel stain (Invitrogen) and was quantified with NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies).

Cellular RNA levels of *CST5*, *SNAI1, SNAI2, TWIST, ZEB1, ZEB2, CDH1* and *LEF-1* were quantified by real-time RT-PCR. The level of *CYP24* RNA was measured in relation to that of *18S* rRNA using the comparative C_T method and RNA TaqMan probes (Applied Biosystems) according to the manufacturer's guidelines and described previously [237], using the 7900HT Fast Real-Time PCR System (Applied Biosystems).

For the synthesis of the first strand of cDNA, 400 ng of total RNA were retro-transcribed using the GeneAmp® Gold RNA PCR Core Kit (Applied Biosystems) that use the MultiScribe *(Murine Leukemia Virus)* Reverse Transcriptase and random hexamers as primers. The reaction was performed in a Light-Cycler apparatus using the LightCycler-FastStart DNA Master SYBR Green I Kit (both from Roche). The final volume in each reaction was 20 μl that

included 2 μl of the retro-transcription product, 0.5 μl of each primer and 1x reaction mix containing FastStar DNA polymerase, reaction buffer, dNTPs and SYBR Green. Thermal cycling for all genes was initiated with a denaturation step of 95ºC for 10 min and consisted of 40 cycles (denaturation at 94ºC 0 s, specific annealing temperature showed in section 4.4 for 5 s, and elongation at 72ºC for 5 s). At the end of each cycle the fluorescence acquisition was made. All the measurements were made in triplicate. At the end of the PCR cycles, melting curve analyses were performed as well as electrophoresis of the products on non-denaturing 8% polyacrylamide gels, followed by sequencing, in order to validate the generation of the specific PCR product expected.

The relative concentration of the target and the reference genes was calculated by interpolation on a standard curve for each gene generated with a serial dilution of cDNA obtained from SW480-ADH cells. Values were normalized versus the geometric average of three control housekeeping genes: *TBP* (TATA binding protein), *SDHA* (succinate dehydrogenase complex subunit A) and *UBC* (ubiquintin C) as described [308].

The sequences of the oligonucleotides used as primers in the quantitative PCR are shown in section 4.4. In all cases the forward and reverse primers were designed in different exons to avoid the genomic DNA amplification that could be contaminating the RNA sample.

The quantitative RT-PCR experiments were performed in collaboration with Dr. F. Bonilla's group from Hospital Universitario Puerta de Hierro, Madrid.

12. Oligonucleotide microarrays

The gene expression patterns of two different clones of HCT116 CST5 and mock cells as well as HCT116 wild-type cells were analyzed using oligonucleotide microarrays (GeneChip Human Gene 1.0 ST Array, Affymetrix). This microarray is designed to measure the gene expression of well-annotated genes, using a single probe set *per* gene comprised of multiple probes that are distributed along the entire length of the genomic locus. Each of the 28,869 genes is represented on the array by approximately 26 probes spread across the full length of the gene. Before the study with microarrays, the expression levels of *CST5* were analyzed by quantitative RT-PCR (described in the previous section) to guarantee the good conditions of the cells and to use them as a control for the future results.

The microarray hibridation as well as the initial processing of data set were performed by the Unidad de Genómica y Proteómica, Centro de Investigación del Cáncer, Salamanca. First, the RNA samples were analyzed with the 2100 Bioanalyzer (Agilent Technologies) to corroborate their quality. Subsequently, 4.4 μg of RNA from each sample were used to synthesize the double strain cDNAs using the SuperScript Double Stranded cDNA Synthesis Kit (Invitrogen). After their purification, the RNA polymerase T7 in presence of biotinylated

nucleotides (BioArray High Yield RNA Transcript Labeling Kit, Enzo Life Science) was used *in vitro* for the transcription of the cDNAs. The biotinylated cRNAs were purified and fragmented to their subsequently microarray hibridization. A fraction of each cRNA was hybridized with a GeneChip Test3 Array (Affymetrix) to verify the label quality. If a favorable result was obtained, each cRNA was hybridized with a GeneChip Human Gene 1.0 ST Array at 45ºC overnight. Later, the microarray was washed and labeled with phycoerythrin-streptavidin and the result was digitalized with a fluorescence scanner.

The fluorescence data were analyzed by the Unidad de Bioinformática, Centro de Investigación del Cáncer, Salamanca. Robust microarray analysis algorithm was used for background correction, intra- and inter-microarray normalization, and expression-signal calculation [31,130,131]. Once calculated, the absolute expression signal for each gene (the signal value for each probe-set) in each microarray, significance analysis of microarrays method [301], was applied to calculate significant differential expression and find the gene probe-sets that characterized the samples of each compared state. The method uses permutations to achieve robust statistical inference of the most significant genes and provides *P* values adjusted to multiple testing using false discovery rate [301]. A cutoff of false discovery rate < 0.05 was used for all the differential expression calculations. Finally, the resulting lists of candidate genes were tested using another algorithm, the so-called global test [93], which reveals the group of genes that has a global expression pattern most significantly related to the clinical feature studied. We applied all these methods using R and Bioconductor.

The comparison of HCT116 CST5 *vs* HCT116 Control candidate genes generated a group of genes downregulated or upregulated by cystatin D overexpression. The classification of differentially expressed genes was carried out filtering the data to include only the transcripts which expression differs at least 1.5-fold between the compared samples.

For functional classification of regulated genes we used Gene Ontology annotations and the information available in the NCBI Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

13. Microarrays validation by quantitative RT-PCR

Validation of selected genes from microarrays data was performed in the Unidad de Genómica del Parque Científico de Madrid.

Total RNA was isolated as previously described in section 11. Cellular RNA levels were measured using the following Taqman probes (Applied Biosystems): *18S rRNA* (Hs99999901_s1), *GAPDH* (Hs99999905_m1), *MAL2* (Hs00294541_m1), *MEF2C* (Hs00231149_m1), *AP1M2* (Hs00194014_m1), *RUNX2* (Hs00231692_m1), *ID3*

(Hs00171409_m1), *TMPRSS4* (Hs00212669_m1), *NAV3* (Hs00372108_m1), *NT5E* (Hs01573922_m1), *VCAN* (Hs00171642_m1), *WNT16* (Hs00365138_m1), *ANX3* (Hs00192982_m1), and *RUNX1* (Hs01021970_m1).

For the synthesis of the first strand of cDNA, 10 ng/μl of total RNA were retro-transcribed using the Applied High Capacity Transcription kit (Applied Biosystems) that use the MultiScribe *(Murine Leukemia Virus)* Reverse Transcriptase and random hexamers as primers. The reaction was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The final volume in each reaction was 10 μl that included 0.5 μl of the retro-transcription product, 0.5 μl of each primer and 250 nM TaqMman probe containing FAM-labeled, MGB-NFQ modified (Applied Biosystems). Thermal cycling for all genes consisted of 2 min at 50ºC, 10 min at 90ºC and 40 cycles (denaturation at 95ºC for 15 s, and elongation at 60ºC for 1 min). All the measurements were made in triplicate.

Data were analyzed using the Sequence Detection System software (Applied Biosystems) and relative RNA levels were calculating using the comparative C_T method as recommended by Applied Biosystems [178].

14. Chromatin immunoprecipitation (ChIP) assays

For chromatin immunoprecipitation assays, the described protocol in the Chromatin Immnunoprecipitation (ChIP) Assay Kit (Upstate) with some modifications [260] was used. Subconfluent cultures of SW480-ADH cells were synchronized with α-amanitin (2.5 μM) in serum-free medium for 2.5 h at 37ºC. After this, cells were rinsed twice in phosphate-buffered saline (PBS) and treated with 1α,25$(OH)_2D_3$ or vehicle during 30 min, 1 or 4 h. Cells were fixed by 15 min of incubation in medium containing 1% of formaldehyde at 37ºC. Subsequently, cells were lysed in an appropriated volume of Lysis Buffer (50 mM TrisHCl pH 8, 10 mM EDTA and 1% SDS plus protease inhibitor cocktail) and chromatin was sheared in a bath sonicator (Diagenode Bioruptor), to an average length of 0.2-1.5 kb. Samples were then centrifuged at 13,000 rpm for 10 min at 4ºC, and the supernatant containing the fragmented chromatin were collected. Two hundred μg of fragmented DNA *per* antibody and condition (including a no-antibody control sample) were diluted in 10x Dilution Buffer (16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100 and 0.01% SDS), pre-cleared for 1 h at 4ºC with 60 μl of a 50% slurry Salmon Sperm DNA/protein G-Sepharose (Upstate), and incubated overnight at 4ºC with antibodies against VDR, SMRT (both from Santa Cruz Biotechnology) histone H4 (acetyl K12) (Upstate) or rabbit IgG (Santa Cruz Biotechnology). Thirty μg of each sample (input) were frozen and kept at -80ºC until the reverse-crosslink step. After 14 h at 4ºC in a rotating platform, samples were incubated in presence of 60 μl of Salmon Sperm DNA-protein A/G Agarose 50% slurry for 1 h. Immune

complexes were eluted after two rounds of incubation with freshly prepared Elution Buffer (1% SDS, 0.1 M $NaHCO_3$), and cross-linking was reverted by addition of 20 μl of 5 M NaCl and incubation at 65ºC for 4 h. After 1 h of proteinase K (Sigma) digestion, DNA was recovered by phenol/chloroform extraction and ethanol precipitation. For semi-quantitative PCR, reactions were performed in a range of amplification that varied from 35 to 38 cycles. The sequence of primers used in these assays is described in section 4.3. For each promoter, the PCR sensitivity was evaluated with input serial dilutions. Amplification products were analyzed by electrophoresis in 2% agarose gel and visualized by SYBR® Safe DNA gel stain (Invitrogen).

15. Western blotting

Whole-cell extracts were prepared by washing the monolayers twice in PBS and cell lysis by incubation in RIPA buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 10% glycerol, 4 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 50 mM Hepes pH 7.4) plus phosphatase- and protease-inhibitor mixture (25 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin) for 15 min on ice followed by centrifugation at 13,000 rpm for 10 min at 4ºC. Protein concentration was measured using the Bio-Rad DC protein assay kit (Bio-Rad). Analysis of cell lysates or immunoprecipitates was performed by electrophoresis in SDS gels and protein transfer to PVDF Immobilon-P membranes (Millipore). The membranes were incubated with blocking solution (5% BSA in TBS 0.1% Tween-20) at room temperature (R/T) and subsequently, with the appropriate primary antibodies prepared in blocking solution (indicated dilutions in section 3) at 4ºC overnight. Finally, the membranes were incubated with the secondary HRP-conjugated antibodies diluted in blocking solution as indicated in section 3 for 1 h at R/T and the antibody binding was visualized using the ECL detection system (Amersham-G.E. Healthcare). The images were acquired with a SNAPSCAN e42 (AGFA) and were quantified with the ImageJ program. In all cases, the shown picture and its quantification belong to a representative experiment of at least three performed.

16. Immunofluorescence and confocal microscopy

Cells were plated in 60 mm or 24-well dishes previously covered with 10 mm diameter cover glasses. At the end of the experiment cells were rinsed once in PBS and fixed in 3.7% formaldehyde for 10 min at R/T. The cells were permeabilized in 0.2% Triton X-100 for 10 min at R/T. Non-specific sites were blocked by incubation with PBS containing 1% Difco™ Skim Milk (BD) for 10 min at R/T before incubating the cells with the appropriated primary

antibodies diluted in PBS for 1 h at 37ºC. After four washes in PBS, the cells were incubated with secondary antibodies for 45 min at R/T, washed three times in PBS and mounted in VectaShield (Vector Laboratories). Propidium iodide (PI) staining was done for 10 min at R/T and followed by four washes in PBS. Images were acquired with an Olympus DP70 digital camera mounted on a Zeiss Axiophot microscope equipped with epifluorescence and confocal images were captured with a Leica TCS SP2 confocal microscope. For double labeling experiments, images of the same confocal plane were generated and superimposed. Phase-contrast images were captured with a Leica DC300 digital camera mounted on an inverted Leitz Labovert FS Microscope. All the images were processed using Adobe Photoshop CS2 software.

The specific primary and secondary antibodies used in these experiments are shown in section 3.

17. Flow cytometry

Cells were synchronized by 17 h incubation with 2.5 mM thymidine (Sigma) in McCoy's 5A medium. Later, they were washed twice in PBS and incubated in normal medium for additional 6 h at 37ºC to release them from the cell cycle blockade. To increase the proportion of G_1-arrested cells, cultures were then treated with 0.5 mM L-mimosine (Sigma) for 20 h. At various timepoints the cells were washed in PBS containing 50 mM EDTA, trypsinized, resuspended in 1 ml PBS/EDTA and fixed by addition of 3 ml ice-cold 100% ethanol and left overnight at 4 ºC. Fixed cells were then pelleted and washed in 1 ml PBS/EDTA, and DNA was stained with 0.025 mg/ml PI (Sigma) in PBS/EDTA containing 0.05% NP40 and 5 ng/µl RNase A. The fraction of the population in each phase of the cell cycle was determined as a function of DNA content using the FACScan flow cytometer FC 500 MPL (Beckman Coulter) equipped with MXP software.

18. Preparation of conditioned media

Cells from 80% confluent 100 mm dishes were washed twice in PBS and incubated in serum-free medium during 48 h. The supernatant were collected and after 10 min centrifugation at 2,000 *g* were concentrated 100x using Amicon Ultra-15 and Microcon YM-10 centrifugal filter units (Millipore Corporation).

19. Cathepsin L activity assays

Cathepsin L activity was assessed using fluorigenic substrate Z-Phe-Arg-AMC (Benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin; Bachem) in the presence of E-64 (Sigma), a broad spectrum inhibitor of cysteine proteases, and CA-074 (Bachem), a specific inhibitor of cathepsin B, by using the Inubushi method [128] with some modifications. Whole-cell extracts were prepared by lysis in cathepsin L lysis buffer (400 mM sodium phosphate buffer pH 6, 75 mM NaCl, 4 mM EDTA, 0.25% Triton X-100), incubated 1 h on ice and homogenized by sonication. Twenty mmol substrate was incubated with the whole-cell extracts in the cathepsin L assay buffer (100 mM sodium acetate buffer pH 5.5, 1 mM EDTA, 2 mM DTT) in the presence of 50 mmol E-64 or CA-074 at 37 ºC for 10 min. The amount of 7-amino-4-methylcoumarin (AMC) liberated from the substrate was monitored fluorometrically with excitation at 370 nm and emission at 480 nm. Total cysteine peptidase activity was determined as the difference between the total activity and the background activity of the non-cysteine peptidases determined by using E-64. Cathepsin L-like activity was measured by inhibiting cathepsin B activity with CA-074. As positive control, recombinant human cystatin D protein (R&D Systems) was used.

20. Cell proliferation assay

To measure proliferation, the cells (8×10^3) were seeded in 24-well plates. After 5 days they were washed once with PBS and living cells were counted after trysinization. In certain experiments the cells were treated for up to 5 days with 100 nM of $1\alpha,25(OH)_2D_3$ or vehicle. Alternatively, [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) assays (Roche Diagnostics) were used. The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. Cells were seeded in 24-well plates. At indicated times, cells were incubated with the MTT solution (final concentration of 0.5 mg/ml) for approximately 4 h at 37ºC. After this incubation period, a water-insoluble formazan dye is formed. After solubilization with 500 µl of 0.04 M HCl isopropanol during 30 min at R/T, the formazan dye was quantitated using a scanning microplate spectrophotometer (VersaMax, Molecular Devices). The absorbance was measured as 570nm-630nm. All experiments were performed at least three times using triplicates.

21. Migration assays

The migratory capacity of HCT116 cells transfected with pcDNA3.1-CST5 or empty vector was analyzed using 8.0 μm pore Transwells® (Corning Inc.). Cells were resuspended in 200 μl of serum-free medium (final concentration of 150,000 cells/ml) and added to the upper side of the Transwell®. At this point, in some experiments, recombinant human cystatin D protein (R&D Systems) or E-64 (Sigma) were added to the resuspended cells medium (at the indicated concentrations). The lower chamber was filled with 600 μl of 20% FBS DMEM or conditioned media from tumor cells. After 24 h incubation, cells attached to the lower surface of the filter were stained using Diff-Quick (Dade Behring) and counted (10 fields/Transwell®) using an inverted Leitz Labovert FS Microscope. Experiments were performed at least three times using duplicates.

22. Anchorage-independent growth assays

HCT116 cells (5 x 10^3) transfected with an empty vector or pcDNA3.1-CST5 were trypsinized and suspended in 1.5 ml of 0.35% Difco Noble agar in DMEM containing 10% FBS. The agar-cell mixture was plated on top of a bottom layer of 0.5% agar (1.5 ml per well in 6-well plates). Plates were incubated at 37ºC in humidified incubator for 14 days. After this time, plates were stained with 0.5 ml of 0.001% Crystal Violet for more than 1 h and viable colonies larger than 50 μm were scored. The experiment was performed in triplicate for each cell line.

23. Xenograft tumor growth

For these assays severe immunodeficient female *scid* mice (B6;CB17-*Ghrhr*^{lit} *Prkdc*^{scid}/BM, The Jackson Laboratories) were used. These mice lack functional T or B-lymphocytes, and tumors from other species are easily transplanted and grow without being rejected. HCT116 or LS174T cells expressing empty vector (mock) or two different clones of pcDNA3.1-CST5 (CST5) were used in these assays. For each cell line, mice were subcutaneously injected with 3 x 10^6 mock or CST5 cells, resuspended in 200 μl of PBS, in left and right flank, respectively. Tumor size was measured 3 times per week by using the ellipsoid volume formula (0.5 x L x W x H) where L, W and H are the tumor length, width and height (in cm), respectively. Animals were euthanized when their external tumor diameter reached 1.5 cm. The maintenance and handling of animals were as recommended by the European Union (ECC Directive of November 24th, 1986, 86/609/EEC) and all experiments were approved by

the Animal Experimentation Committee of our Institute. Every effort was made to minimize animal suffering and to reduce the number of animals used.

24. Immunohistochemistry

Human tissues were obtained as formalin-fixed, paraffin-embedded tissue sections from the archives of the tumor bank from the Hospital Universitario Central de Asturias, Oviedo. They were made anonymously according to the guidelines approved by the Research Ethics Board of the Hospital. Tissue arrays containing a total of 51 samples including three replicates representing different locations were used to evaluate cystatin D and VDR expression according to the differentiation status. After dewaxing and rehydrating, samples were blocked in 15% goat serum and then incubated overnight at 4ºC with anti-cystatin D (1:1,000) or anti-VDR antibody (1:150). Visualization of specific interactions was monitored by using the EnVision HRP System (DAKO, Copenhagen, Denmark) following the manufacturer's instructions, and the staining was completed by incubation with diaminobenzidine colorimetric reagent (DAKO), followed by counterstaining with hematoxylin. Finally, the slides were dehydrated and mounted. Controls included samples that were incubated with a preimmune serum. Normal parotid tissue was used as positive control for cystatin D. Protein expression was graded independently by two observers as either very high (+++), high (++), moderate (+), low (+/-) or negative (-) depending on the level of epithelial staining.

Formalin-fixed, paraffin-embedded tissue sections from tumors generated by SW480-ADH cells in immunodeficient mice that were treated with EB1089 (100 nM) or placebo [226] were immunostained following the previously described protocol.

This study was performed in collaboration with Dr. Aurora Astudillo and Marta S. Pitiot from the Hospital Universitario Central de Asturias, Oviedo.

25. Quantification of protein expression in human samples

Tissue protein from normal and tumor tissue samples (described in section 2) was extracted by pulverizing the samples in liquid nitrogen using a mortar and homogenization using a Potter-Elvehjem apparatus in lysis buffer (50 mM Tris-HCl pH 7.5, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 10% glycerol, 0.5 mM DTT and protease and phosphatase inhibitor mix) on ice. After 20 min centrifugation at 13,000 rpm, the expression level of cystatin D, VDR and E-cadherin proteins was analyzed by western blotting.

26. Statistical analysis

The data are expressed as the mean ± standard deviation (SD) unless otherwise specified. Statistical significance was assessed by two-tailed unpaired student's *t*-test. The single asterisk indicates $P < 0.05$, the double asterisk $P < 0.01$, and the triple asterisk $P < 0.001$. When $P > 0.05$, the data were considered not significant (ns). All statistical analyses were performed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). As the tumor/normal tissue (T/N) ratios of VDR and cystatin D expression were not normally distributed (Kolmogorov-Smirnov test, Lilliefors correction), the data distribution was normalized using $\log_2$ for statistical analysis [226]. The geometric (rather than the arithmetic) average of the T/N ratio was used. Correlations between protein expression levels were analyzed using the Spearman correlation coefficient. In addition, cystatin D protein expression was divided in two groups, low and high expression, according to the median value of this variable and the expression of VDR and E-cadherin in these two groups was represented in a box-plot graphic. The comparison between the two groups of data was done using the Kruskal-Wallis test.

RESULTS

1. 1α,25(OH)$_2$D$_3$ increases cystatin D RNA and protein expression

Previous studies of our group analyzing the gene expression profile induced by 1α,25(OH)$_2$D$_3$ in SW480-ADH colon cancer cells showed the regulation of around 250 genes ([227] and unpublished data). Approximately, two thirds of these genes are upregulated by the hormone, while one third are inhibited. One of the 1α,25(OH)$_2$D$_3$-upregulated genes is *CST5*, that encodes cystatin D, whose RNA levels increased 3.73-fold after 4 h of treatment.

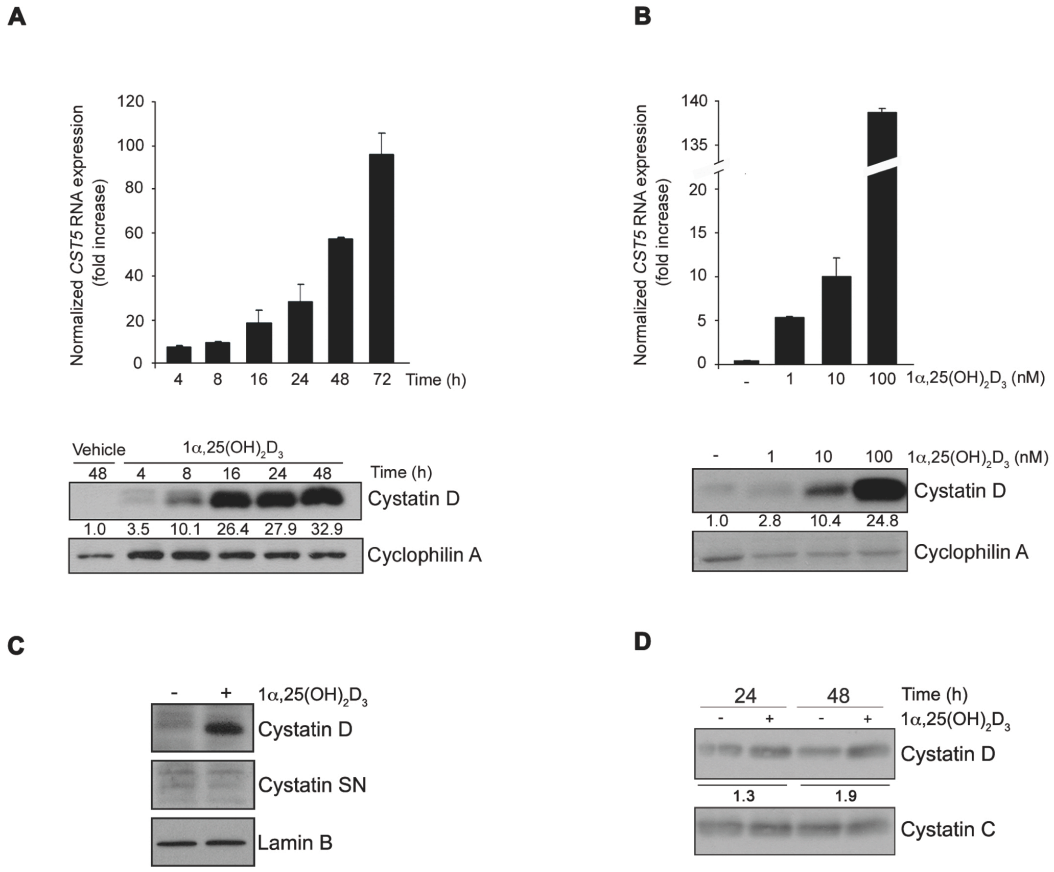

Figure 10. 1α,25(OH)$_2$D$_3$ induces cystatin D expression in SW480-ADH cells. (A) Kinetics of *CST5* RNA (top) and protein (bottom) induction by 1α,25(OH)$_2$D$_3$. SW480-ADH cells were incubated with 1α,25(OH)$_2$D$_3$ (100 nM) or vehicle for the indicated times, and the levels of *CST5* RNA and protein were measured by quantitative RT-PCR (top) or western blot (bottom) as explained in Methods. Normalized mean values and SD obtained in three independent experiments are shown. **(B)** Dose-curve induction of *CST5* RNA (top) and protein (bottom) by 1α,25(OH)$_2$D$_3$. **(C)** Lysates of cells treated with 1α,25(OH)$_2$D$_3$ (100 nM) for 48 h were analyzed by western blot with antibodies against cystatin D or cystatin SN. **(D)** Conditioned media of cells treated or not with 1α,25(OH)$_2$D$_3$ (100 nM) for 24 or 48 h were analyzed by western blot with antibodies against cystatin D or cystatin C. Numbers between the blots correspond to mean of the fold increase values obtained in two experiments.

To validate the upregulation of *CST5* by 1α,25(OH)$_2$D$_3$ we performed quantitative RT-PCR and western blot analyses. Kinetics analyses of the cellular RNA and protein content following 1α,25(OH)$_2$D$_3$ addition (100 nM) showed increased *CST5* RNA and protein level with a gradually increase, reaching around 60- and 30-fold, respectively, at 48 hours post-treatment (Figure 10A). This effect was dose-dependent and physiological concentrations

(10 and 1 nM) of 1α,25(OH)$_2$D$_3$ caused 2.8- and 10.4-fold-increase of protein expression level, respectively. Similar results were observed at RNA level (Figure 10B). The induction of cystatin D by 1α,25(OH)$_2$D$_3$ was specific, as no induction of cystatin SN was found (Figure 10C). We also analyzed if 1α,25(OH)$_2$D$_3$ regulated the amount of secreted cystatin D in the medium. Indeed, the level of secreted cystatin D protein increased slightly at 24-48 hours post-treatment (2-fold; Figure 10D), while no change in cystatin C was observed. Next, we examined if 1α,25(OH)$_2$D$_3$ upregulates cystatin D in other colon cancer cell lines. By quantitative RT-PCR we found an induction of cystatin D expression in Caco-2, LS174T, Colo205, and HCT116 cells in good correlation with the activation of a consensus VDRE (Figure 11A and 11B).

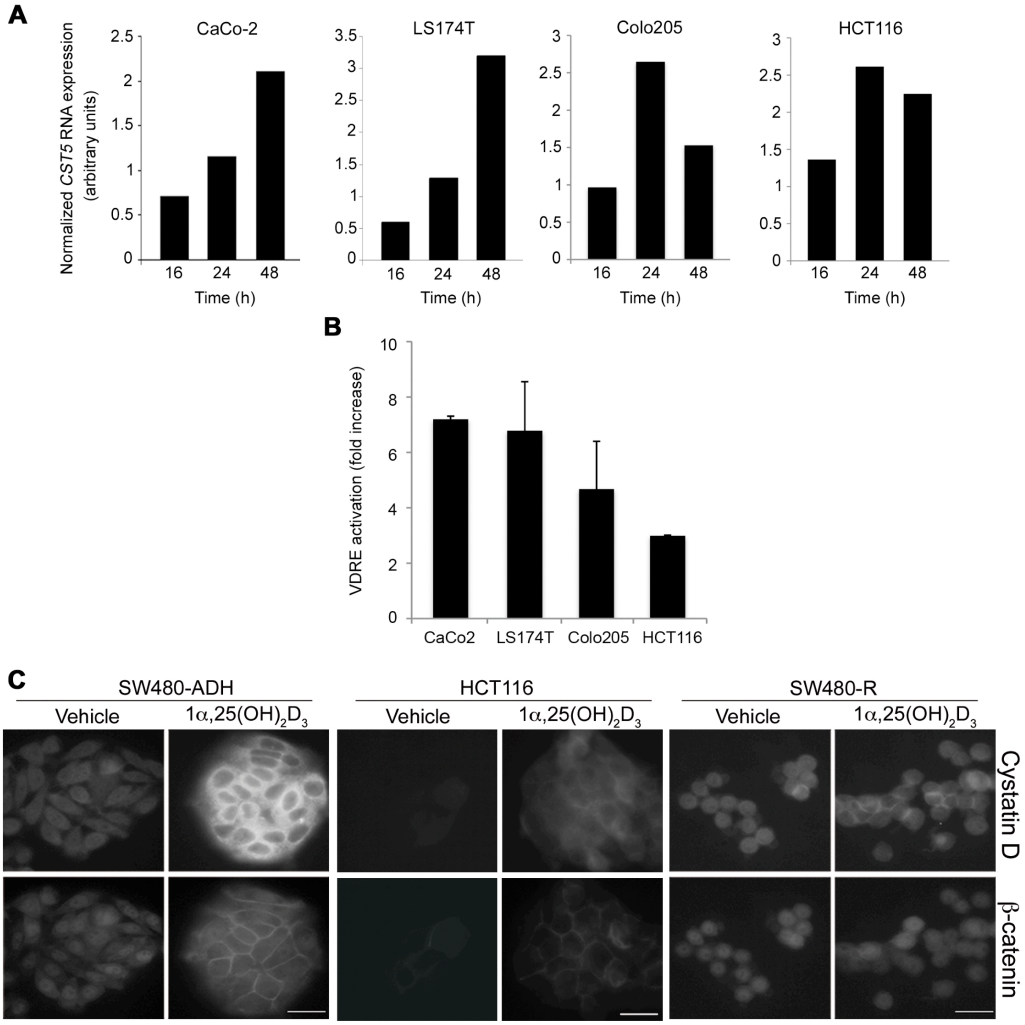

Figure 11. 1α,25(OH)$_2$D$_3$ induces cystatin D expression in several human colon cancer cell lines. (A) Normalized level of *CST5* RNA in CaCo-2 (left), LS174T (middle left), Colo205 (middle right) and HCT116 (right) cells treated with 1α,25(OH)$_2$D$_3$ (100 nM) for the indicated times as estimated by quantitative RT-PCR. **(B)** Ligand-activation of a consensus vitamin D response element in CaCo-2, LS174T, Colo205 and HCT116 cells. Cells were transfected with the 4xVDRE-DR3-Tk-Luc contruct and 24 h later were incubated with 1α,25(OH)$_2$D$_3$ (100 nM) or vehicle for additional 48 h. Values correspond to luciferase induction by 1α,25(OH)$_2$D$_3$ in three independent experiments done in triplicate. **(C)** Immunofluorescence analysis of cystatin D induction. Images of SW480-ADH (left), HCT116 (middle) and SW480-R (right) cells treated with 1α,25(OH)$_2$D$_3$ (100 nM) or vehicle for 48 h. The relocalization of β-catenin was analyzed as control of 1α,25(OH)$_2$D$_3$ action. Scale bars: 20 μm.

The increase in cystatin D protein was confirmed by immunofluorescence studies, which also showed a predominant localization of cystatin D in the cytoplasm of SW480-ADH cells treated with 1α,25(OH)$_2$D$_3$ (Figure 11C, left panel). The relocation of β-catenin from the nucleus and cytosol towards the plasma membrane was used as control of 1α,25(OH)$_2$D$_3$ activity. This induction, although weaker than in SW480-ADH, was also observed in HCT116, whereas no cystatin D was detected in SW480-R cells that contain very low VDR levels and do not respond to 1α,25(OH)$_2$D$_3$ (Figure 11C, middle and right panels).

To analyze whether the regulation of cystatin D expression takes place *in vivo*, we performed immunohistochemical analyses of tumors generated by SW480-ADH cells in immunodeficient mice that were treated with EB1089, a less calcemic 1α,25(OH)$_2$D$_3$ analog [226]. We analyzed several samples and performed the quantification of cystatin D expression by estimation of staining intensity as described in Materials and Methods section. In line with *in vitro* data, EB1089 treatment increased cystatin D expression, and thus, 80% of samples of mice treated with EB1089 showed high/very high staining level, whereas in control mice treated with placebo only 33.3% of samples were high/very high stained (Figure 12).

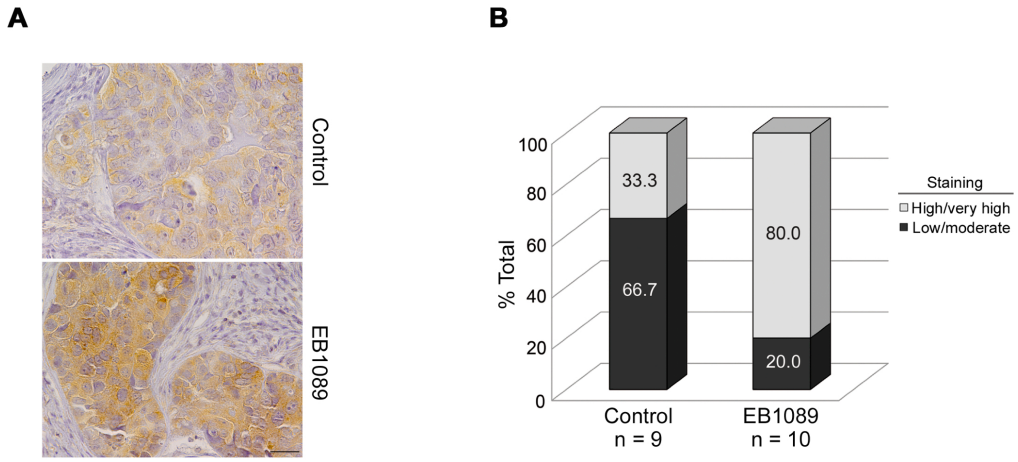

Figure 12. The 1α,25(OH)$_2$D$_3$ analog EB1089 induces cystatin D expression *in vivo*. **(A)** Immunohistochemical analysis of cystatin D expression in tumors generated by SW480-ADH cells in immunodeficient mice that were treated with EB1089 (100 nM) or placebo. Scale bar: 200 μm. **(B)** Quantification of cystatin D expression by estimation of staining intensity as described in Methods. The number of samples analyzed *per* group and the percentage corresponding to each level of cystatin D staining are shown.

To assess the functionality of the cystatin D protein induced by 1α,25(OH)$_2$D$_3$ we studied the activity of its cathepsin targets in SW480-ADH cells (Figure 13). A series of enzymatic assays using specific substrates for two different cathepsins (L and B), showed that cathepsin L activity in extracts from 1α,25(OH)$_2$D$_3$-treated cells was lower (~ 40%) than in those of vehicle-treated cells. Total cathepsin activity also decreased (~ 50%), whereas that of cathepsin B, that is not inhibited by cystatin D, was unchanged by 1α,25(OH)$_2$D$_3$.

Recombinant cystatin D protein was used as control. No changes were found in total extracellular cathepsin activity (data not shown).

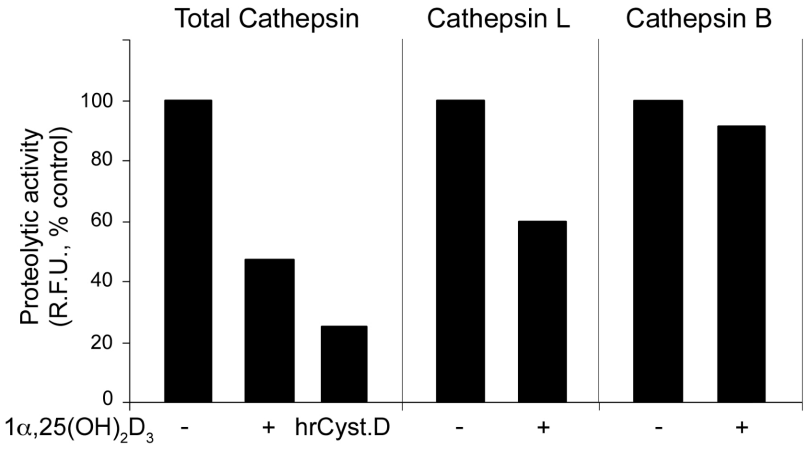

Figure 13. Effect of 1α,25(OH)$_2$D$_3$ on cathepsin enzymatic activity. 1α,25(OH)$_2$D$_3$ inhibits cathepsin activity in SW480-ADH cells. Enzymatic assays for total cathepsin activity and for that of cathepsin L and cathepsin B in extracts from vehicle- and 1α,25(OH)$_2$D$_3$-treated cells were performed as described in Methods. Human recombinant cystatin D (hrCyst.D; 5 μg/ml) protein was used as control. Specific activity was expressed as relative fluorescence units (RFU).

Altogether, these data validate the results obtained in the gene expression profile studies, and confirme cystatin D as a 1α,25(OH)$_2$D$_3$ target, not only in SW480-ADH cells but also in other colon cancer cell lines.

2. 1α,25(OH)$_2$D$_3$ induces cystatin D expression by direct activation of its gene promoter

We next examined the mechanism by which 1α,25(OH)$_2$D$_3$ induced *CST5* RNA and protein expression. For this reason we used the transcription and translation inhibitors actinomycin D and cycloheximide, respectively, in combination with 1α,25(OH)$_2$D$_3$ or vehicle (Figure 14). By quantitative RT-PCR we observed that the increase in *CST5* RNA was abrogated by actinomycin D but not ($P > 0.05$) by cycloheximide, indicating a direct transcriptional effect of 1α,25(OH)$_2$D$_3$.

These results led us to study the effect of 1α,25(OH)$_2$D$_3$ on *CST5* gene promoter. The *in silico* analysis of 2.1 kb (-1867/+262) *CST5* gene promoter sequence allowed the identification of several putative VDR binding hemisites (consensus RGKTCA; R = A or G, K = G or T) that can be grouped in four (A to D) regions (Figure 15).

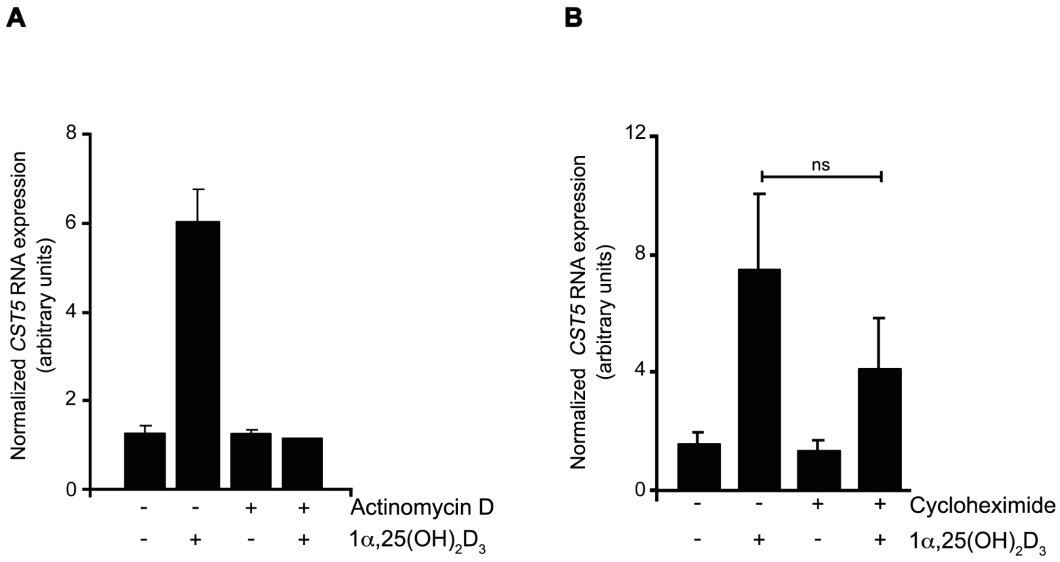

Figure 14. Direct transcriptional effect of 1α,25(OH)$_2$D$_3$ on cystatin D expression. Quantitative RT-PCR analysis of *CST5* RNA expression in SW480-ADH cells treated with 1α,25(OH)$_2$D$_3$ or vehicle that were pretreated for 30 minutes with **(A)** actinomycin D (2 μg/ml) or **(B)** cycloheximide (8 μg/ml) as indicated.

To identify which hemisites could be responsible for 1α,25(OH)$_2$D$_3$ effect we generated four different fragments of the promoter (-1867/, -1128/, -650/ and -251/+262) containing one, five, seven or eight putative VDREs, respectively. These fragments were cloned by PCR into the pGL3 luciferase expression vector.

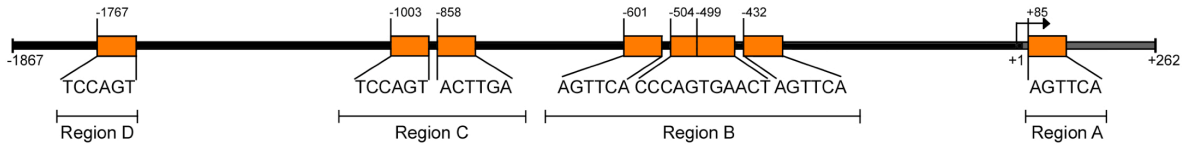

Figure 15. Scheme of the proximal human *CST5* gene promoter. Putative VDR binding site sequences and their position grouped in regions A-D are shown.

Transactivation assays in SW480-ADH cells showed that, actually, 1α,25(OH)$_2$D$_3$ activates the *CST5* promoter. Moreover, these assays revealed that most of the activation depends on the cluster of sites in region B (-650/-262) with no contribution of that in region A (Figure 16A, left panel). We also analyzed if this activation took place in other colon cancer cell lines. Indeed, *CST5* promoter activation was also observed in LS174T and HCT116 cell lines but not in the VDR-deficient SW480-R or HEK293T cells (Figure 16B). However, the expression in HEK293T cells of an exogenous functional VDR led to an induction of *CST5* promoter, similar to that observed in SW480-ADH cells (Figure 16A, right panel).

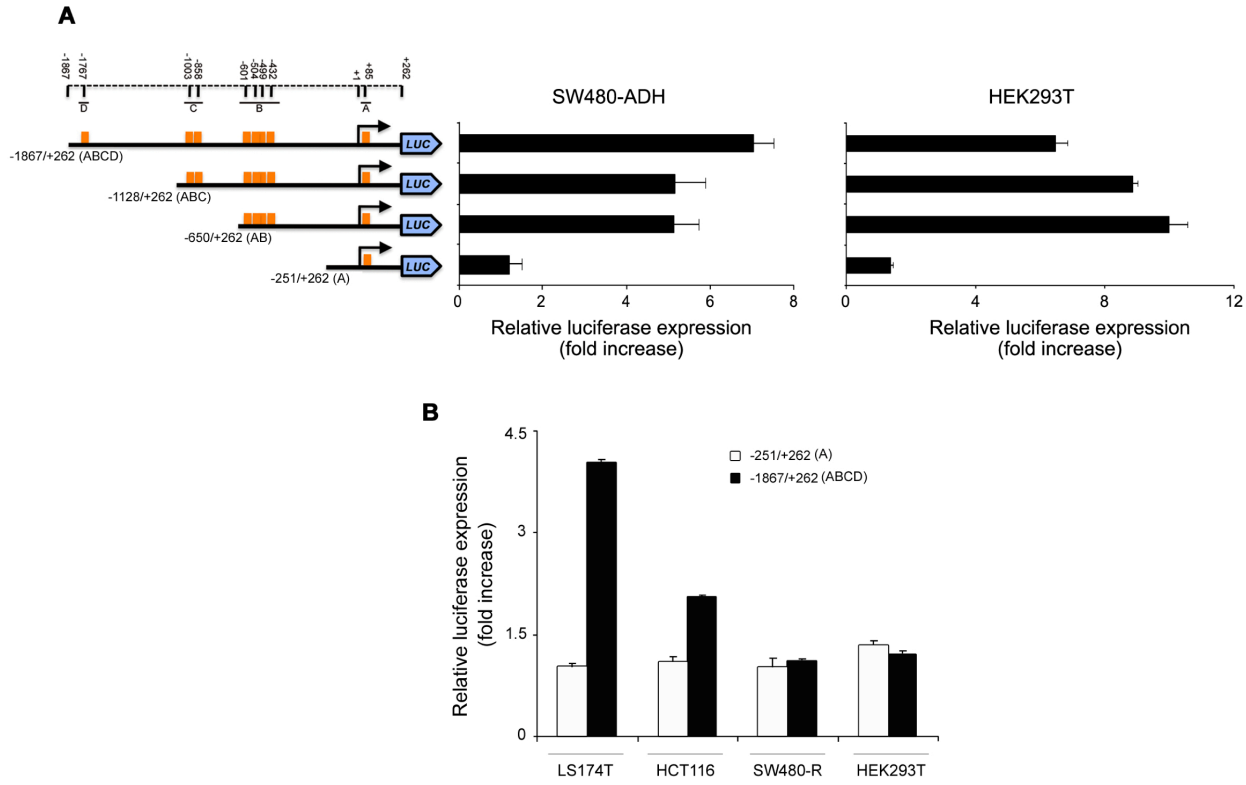

Figure 16. 1α,25(OH)$_2$D$_3$ directly activates the *CST5* promoter. **(A)** Activation of *CST5* promoter constructs by 1α,25(OH)$_2$D$_3$ in SW480-ADH cells (left) and HEK293T cells cotransfected with an expression vector for wild-type VDR (right). **(B)** Activation of *CST5* promoter by 1α,25(OH)$_2$D$_3$ in LS174T and HCT116 cells. VDR deficient SW480-R and HEK293T cells were used as negative control. Twenty-four hours after transfection, cells were treated with vehicle or 1α,25(OH)$_2$D$_3$ (100 nM) for an additional 48 h. The empty pGL3 vector was used as control. Values correspond to promoter induction by 1α,25(OH)$_2$D$_3$ in three independent experiments done in triplicate.

In addition, experiments using SW480-R cells transfected with either wild-type VDR or the transcription activation-deficient VDR$^{\Delta AF2}$ mutant assessed the requirement of a functional VDR for the activation of the *CST5* promoter by 1α,25(OH)$_2$D$_3$ (Figure 17). These results show that 1α,25(OH)$_2$D$_3$ induces cystatin D expression at the transcriptional level.

To explore if the transcriptional activation of *CST5* by 1α,25(OH)$_2$D$_3$ is mediated by a direct mechanism (implying the binding of VDR to its promoter), we performed chromatin immunoprecipitation (ChIP) assays (Figure 18). We used specific oligonucleotides that include the region B (-840/-571), and as negative control oligonucleotides including the region A (-63/+66). Binding of VDR to the -840/-571 fragment was already observed at 30 min after treatment with 1α,25(OH)$_2$D$_3$ and it was maintained after 4 h. No binding was detected to region A (data not shown). This event took place in parallel with the release of SMRT corepressor from the promoter and an increase in histone H4 acetylation, a marker of transcriptional activation. As a positive control we used *CYP24*, a target gene of 1α,25(OH)$_2$D$_3$ that is induced directly by the binding of VDR to its promoter.

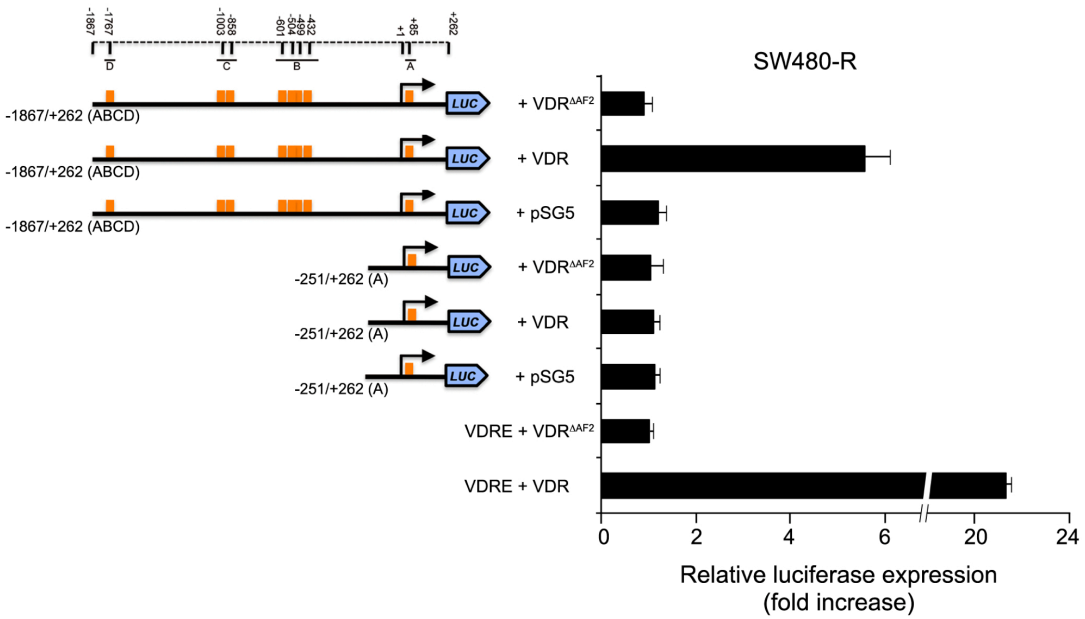

Figure 17. The activation of the *CST5* promoter by 1α,25(OH)$_2$D$_3$ requires a transcriptionally competent VDR. SW480-R cells were cotransfected with the promoter construct pGL3-1867 or pGL3-251 and either the wild-type VDR or the mutant ΔAF2-VDR (VDR$^{\Delta AF2}$), or an empty vector. The cells were then treated with 1α,25(OH)$_2$D$_3$ (100 nM) or vehicle for 48 h. As control the consensus 4xVDRE-DR3-Tk-Luc (VDRE) reporter construct was cotransfected with the wild-type or mutant VDR. Values correspond to promoter induction by 1α,25(OH)$_2$D$_3$ in three independent experiments done in triplicate.

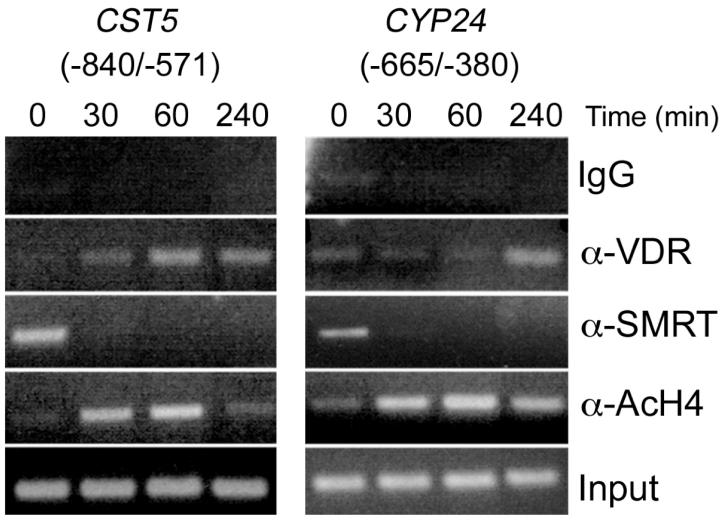

Figure 18. 1α,25(OH)$_2$D$_3$ induces VDR binding to and an active chromatin conformation of the *CST5* promoter *in vivo*. ChIP assay showing the induction by 1α,25(OH)$_2$D$_3$ of VDR binding, SMRT corepressor release, and increased histone H4 acetylation (AcH4) of the *CST5* gene promoter in SW480-ADH cells. The *CYP24* gene was used as control. The promoter regions studied are indicated.

Altogether, these results show that *CST5* is a direct transcriptional target of 1α,25(OH)$_2$D$_3$.

3. Acidic retinoids synergize with 1α,25(OH)$_2$D$_3$ to enhance cystatin D expression

Retinoids are structural and functional analogs of vitamin A. They are involved in the modulation of several biologic functions such as regulation of cell proliferation and differentiation, growth of bone tissue, immune function, and activation of tumor suppressor genes [280]. Retinoids exert their pleiotropic effects through the interaction with nuclear receptors, defined as retinoic acid receptors (RARs) and retinoid X receptors (RXRs). These ligand-activated nuclear receptors induce the transcription of target genes by binding to retinoic acid (RA) responsive elements (RAREs) present in promoter regions. RARs and RXRs are also capable to interact with other nuclear receptors, and thus RARs can heterodimerize with RXRs, while RXRs heterodimerize with other nuclear receptors, including the thyroid hormone receptor (TR), vitamin D receptor (VDR), and peroxisomal proliferator activated receptor (PPAR), among others [42].

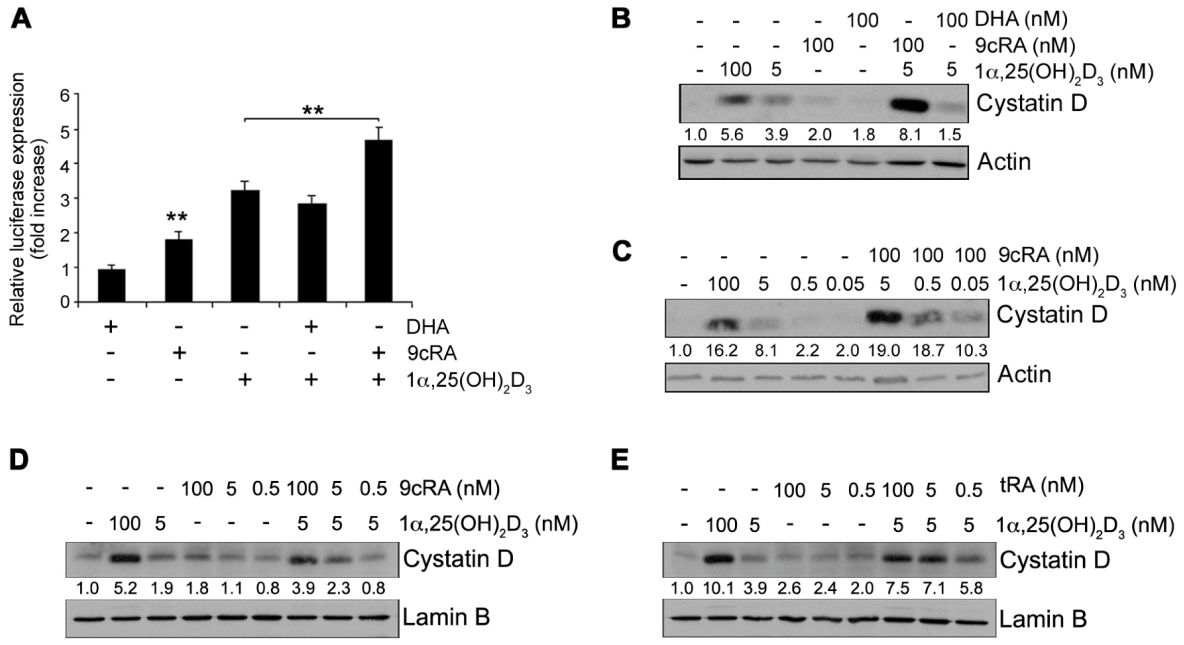

Figure 19. Acidic retinoids potentiate 1α,25(OH)$_2$D$_3$ induction of cystatin D expression. **(A)** Activation of *CST5* promoter construct by DHA or 9cRA alone or in combination with 1α,25(OH)$_2$D$_3$ in SW480-ADH cells. The cells were transfected with the promoter construct pGL3-650 or an empty vector. Twenty-four hours after transfection, cells were treated with vehicle, 1α,25(OH)$_2$D$_3$ (100 nM), DHA (100 nM), 9cRA (100 nM) or a combination of DHA or 9cRA with 1α,25(OH)$_2$D$_3$ for and additional 48 h. Values correspond to promoter induction in three independent experiments done in triplicate. **(B)** Lysates of cells treated during 48 h with DHA, 9cRA or 1α,25(OH)$_2$D$_3$ at the indicated concentrations and combinations were analyzed by western blot. **(C)** Western blot showing changes in cystatin D protein in SW480-ADH cells treated with 1α,25(OH)$_2$D$_3$ at the indicated concentrations alone or in combination with 9cRA. **(D)** 9cRA-mediated potentiation of cystatin D expression by 1α,25(OH)$_2$D$_3$ is dose-dependent. **(E)** tRA synergizes with 1α,25(OH)$_2$D$_3$ to enhance cystatin D expression in a dose-dependent fashion. Lysates of SW480-ADH cells treated during 48 h with 1α,25(OH)$_2$D$_3$ and/or 9cRA or tRA at the indicated concentrations were analyzed by western blot. Numbers between the blots in **(B-E)** correspond to mean of the fold increase values obtained in two experiments.

Given that the interaction between VDR and RXR in transcription regulation is well known, we investigated if any RXR ligands could cooperate with 1α,25(OH)$_2$D$_3$ for cystatin D induction. First, we analyzed *CST5* promoter induction using 9-*cis* retinoic acid (9cRA), the most recognized RXR ligand, as well as docosahexaenoic acid (DHA), an omega-3 fatty acid that has been described as a natural ligand of RXR in mouse brain [55]. We transfected the -650/+262 *CST5* promoter fragment in SW480-ADH cells and treated the cells with 1α,25(OH)$_2$D$_3$ in combination with 9cRA or DHA for 48 h (Figure 19A). *CST5* promoter was slightly induced by 9cRA (1.8-fold). Importantly, the combination of 9cRA with 1α,25(OH)$_2$D$_3$ caused a stronger induction of the promoter than that observed with 1α,25(OH)$_2$D$_3$ alone (4.6-fold *versus* 3.2-fold). By contrast, DHA treatment did not activate *CST5* promoter alone nor synergized with 1α,25(OH)$_2$D$_3$.

Next, we examined whether cystatin D protein expression was modified by RAs. SW480-ADH were treated with different concentrations of 1α,25(OH)$_2$D$_3$ (100 or 0.5 nM) in combination with 9cRA (100 nM) or DHA (100 nM) during 48 h and analyzed by western blot. Similarly to data obtained in the promoter studies, the induction by 1α,25(OH)$_2$D$_3$ of cystatin D protein expression increased following the combined treatment with 9cRA but not with DHA (Figure 19B). An extended study using lower concentrations of 1α,25(OH)$_2$D$_3$ corroborated that 9cRA enhances the induction of cystatin D by 1α,25(OH)$_2$D$_3$ (Figure 19C).

We also analyzed if *all*-trans retinoic acid (tRA), regulates cystatin D expression. tRA binds and activates RAR, and has no effect over RXR. Western blot analysis after treatment with different concentrations of 1α,25(OH)$_2$D$_3$ and 9cRA, tRA or the combination of 9cRA or tRA with 1α,25(OH)$_2$D$_3$ showed that, similarly to 9cRA, tRA increases the induction of cystatin D by 1α,25(OH)$_2$D$_3$. Moreover, treatment with different concentrations of 9cRA and tRA (100, 5 and 0.5 nM) demonstrated that the effect of both RAs was dose-dependent (Figure 19D and 19E).

These results show that the induction of cystatin D by 1α,25(OH)$_2$D$_3$ is enhanced by both RXR and RAR ligands.

4. Ectopic expression of cystatin D in SW480-ADH cells mimics partially the effects of 1α,25(OH)$_2$D$_3$

To examine the contribution of cystatin D to the antitumor action of 1α,25(OH)$_2$D$_3$ we expressed ectopically the human cystatin D cDNA in SW480-ADH cells. They were selected due to its high response to 1α,25(OH)$_2$D$_3$ treatment, and because these cells are widely used as a CRC model since they harbor most of the frequent mutations found in this neoplasia (CIN phenotype; wild-type *CTNNB1*/β-catenin, *TGFβRII* and *BAX,* mutated *TP53, APC* and

K-RAS). Expression of exogenous cystatin D protein in transfected clones following antibiotic selection was analyzed by western blotting and two clones (#8 and #17) with different cystatin D expression levels were selected for further studies. The activity of exogenous cystatin D was assessed in enzymatic assays (Figure 20).

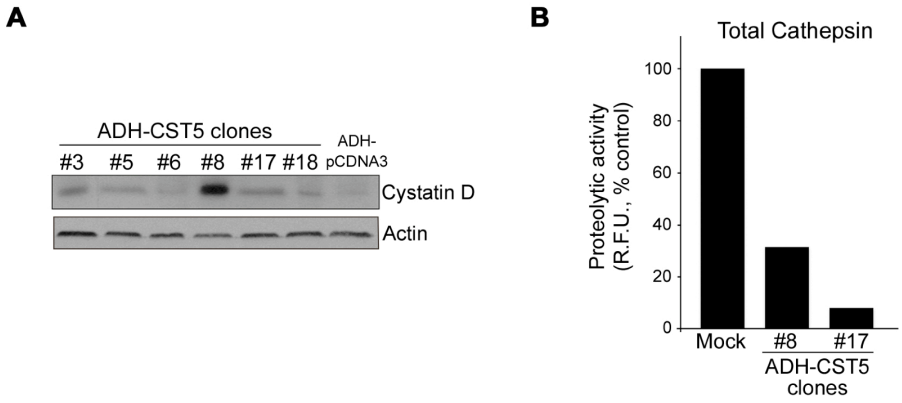

Figure 20. Ectopic cystatin D expression in stably-transfected SW480-ADH cells. **(A)** Western blot analysis showing the expression of cystatin D protein in G418-selected clones of transfected SW480-ADH cells. **(B)** Total cathepsin activity in selected SW480-CST5 clones. Specific activity was expressed as relative fluorescence units (RFU).

1α,25(OH)$_2$D$_3$ inhibits the proliferation of SW480-ADH cells through the regulation of multiple cell cycle-related proteins and the inhibition of β-catenin/TCF4 transcriptional activity [77,225,303]. These findings led us to examine if ectopic cystatin D expression altered the proliferation capacity of SW480-ADH cells. For this purpose, CST5-expressing cells were treated with 1α,25(OH)$_2$D$_3$ or vehicle. We observed that exogenous cystatin D decreased cell proliferation in a comparable level to that of 1α,25(OH)$_2$D$_3$ and their combination had an additive effect (Figure 21).

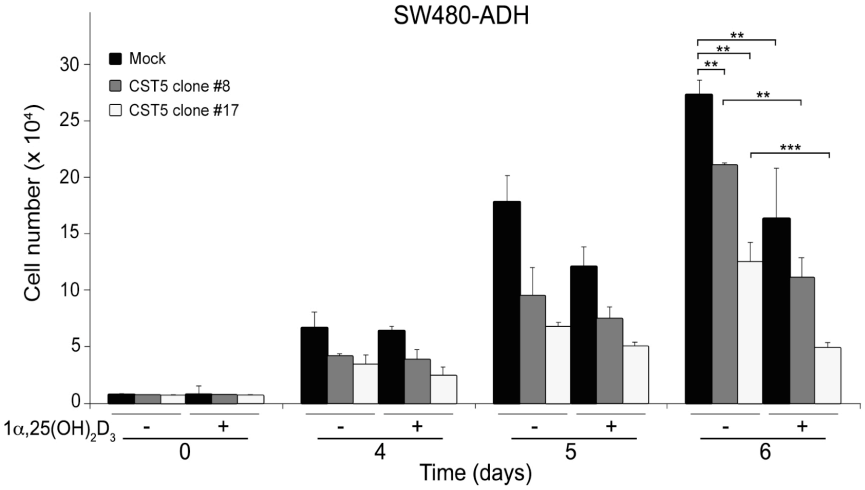

Figure 21. Ectopic cystatin D expression inhibits proliferation in SW480-ADH cells. SW480-ADH cells transfected with empty vector (Mock) or stably expressing CST5 were seeded in 24-well plates and treated for up to 6 days with 1α,25(OH)$_2$D$_3$ (100 nM) or vehicle. Living cells were counted after trypsinization. Representative data from three independent experiments done in triplicate are shown.

Next, we studied if SW480-ADH gene expression was modified by exogenous cystatin D. We analyzed several 1α,25(OH)$_2$D$_3$ target genes as *CYP24* and *CDH1*. In both cases exogenous cystatin D enhanced the induction by 1α,25(OH)$_2$D$_3$ (1.4- and 2.5-fold increase of RNA levels, respectively) (Figure 22A left and middle panels). In case of E-cadherin, this increased induction was also observed at the protein level (1.6-fold) (Figure 22B).

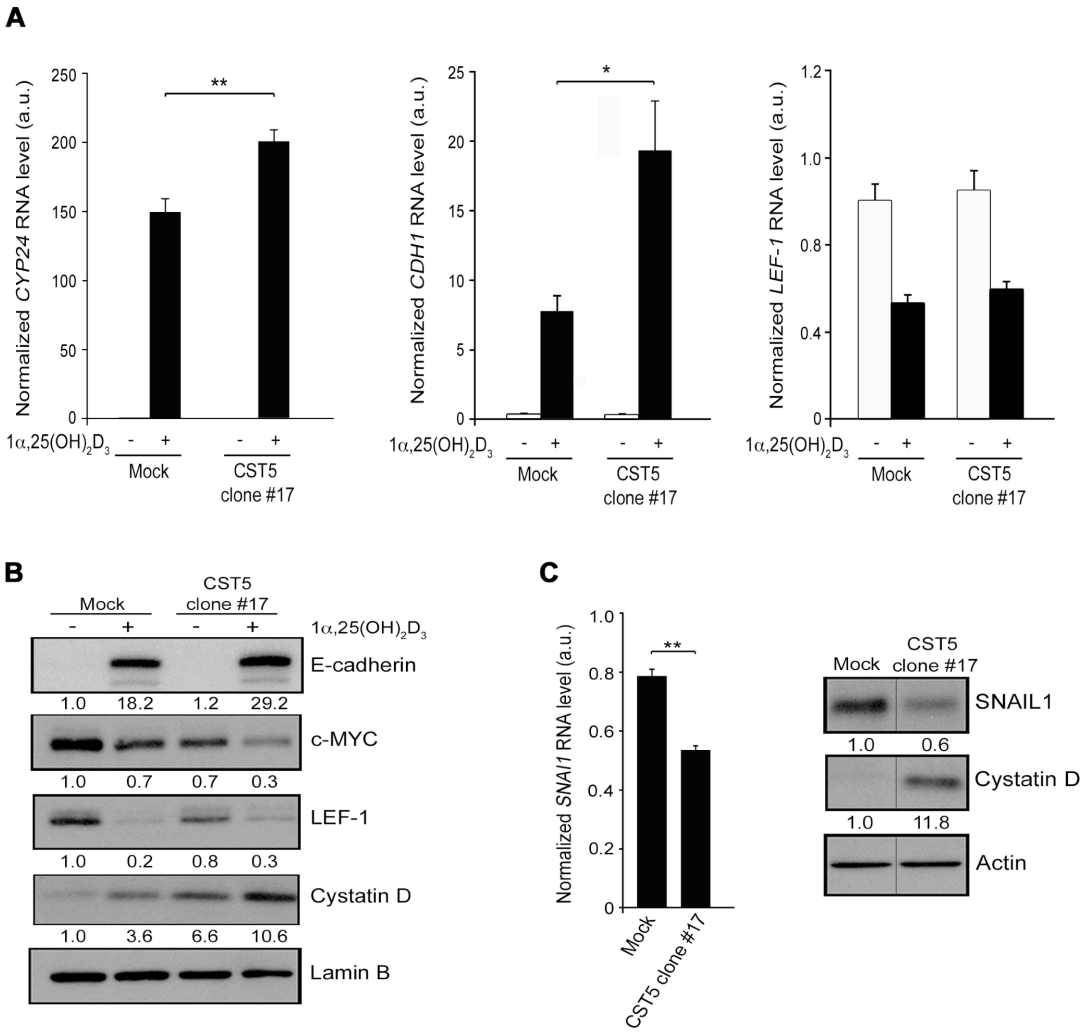

Figure 22. Ectopic cystatin D expression alters gene expression in SW480-ADH cells. **(A)** Quantitative RT-PCR analysis showing the level of *CYP24* (left), *CDH1* (middle), and *LEF1* (right) RNA in cystatin D-expressing cells. **(B)** Western blot analysis showing changes in E-cadherin, c-MYC, and LEF-1 proteins in cystatin D-expressing cells. **(C)** Cystatin D inhibits *SNAI*1 expression. Quantitative RT-PCR (left) and western blot (right) analyses showing reduced *SNAI1* RNA and protein levels in cystatin D-expressing cells. Numbers between the blots in **(B and C)** correspond to mean of the fold change values obtained in two experiments.

As cystatin D inhibited cell proliferation we analyzed the expression of *c-MYC*, an oncogene that controls several cell proliferation related genes, acting as a key cell cycle regulator. Moreover, *c-MYC* is downregulated by 1α,25(OH)$_2$D$_3$ [34,228,229]. We observed that cystatin D-expressing cells showed a reduced basal level of c-MYC protein expression. Furthermore, exogenous cystatin D enhanced the repression of c-MYC by 1α,25(OH)$_2$D$_3$

(57% in cystatin D-expressing cells *versus* 30% in mock cells) (Figure 22B). Notably, exogenous cystatin D decreased RNA and protein levels of the EMT inducer SNAIL1 (37.5% and 40% respectively) (Figure 22C). We also studied the effect of ectopic cystatin D expression on the mesenchymal marker LEF-1 level. We analyzed RNA and protein expression but no significant change was observed (Figure 22A, right panel and 22B).

These data suggest a possible role of cystatin D as a mediator of some $1\alpha,25(OH)_2D_3$ antitumor actions.

5. *CST5* silencing affects the response of SW480-ADH to $1\alpha,25(OH)_2D_3$

To further investigate the effects of cystatin D on the behavior of SW480-ADH cells after $1\alpha,25(OH)_2D_3$ treatment and elucidate if this protease inhibitor has an important role in the hormone activity, we knocked-down cystatin D by transduction of stable lentiviral shRNA. Control cells were infected with a nontargeting shRNA. Western blot analysis showed that cystatin D levels in *CST5* shRNA cells were severely reduced as compared to control following $1\alpha,25(OH)_2D_3$ addition (Figure 23A).

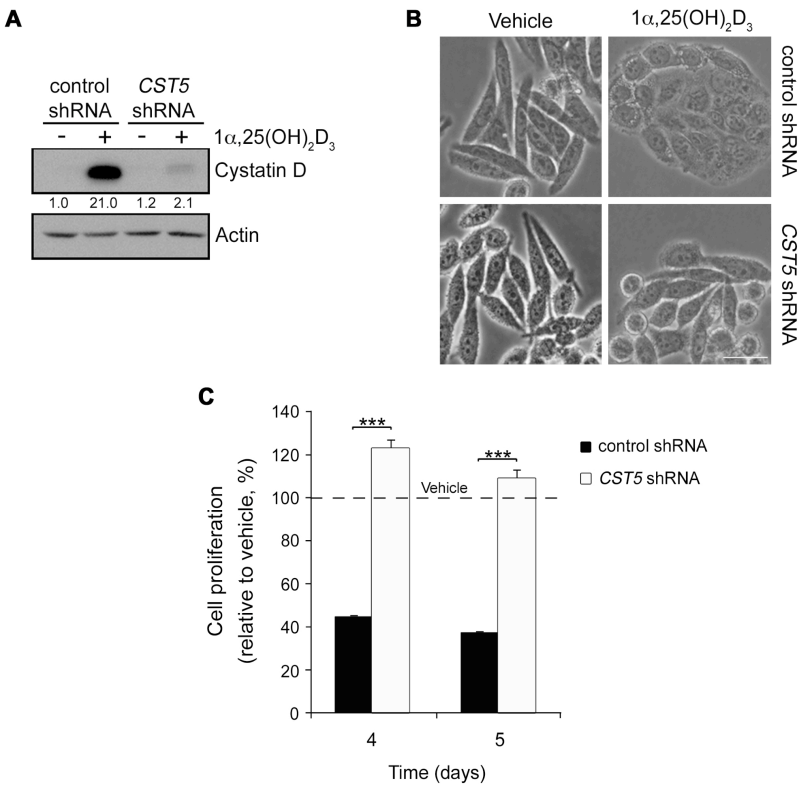

Figure 23. *CST5* silencing affects SW480-ADH response to $1\alpha,25(OH)_2D_3$. **(A)** Western blot analysis showing decreased level of cystatin D protein in *CST5* shRNA cells treated with $1\alpha,25(OH)_2D_3$ (100 nM) or vehicle. Numbers between the blots correspond to mean of the fold change values obtained in two experiments. **(B)** Phase-contrast images of control shRNA and *CST5* shRNA cells that were treated with $1\alpha,25(OH)_2D_3$ (100 nM) or vehicle for 48 h. Scale bar: 20 μm. **(C)** *CST5* knock-down abrogates the inhibition of cell proliferation by $1\alpha,25(OH)_2D_3$. Control shRNA and *CST5* shRNA cells were treated with vehicle or $1\alpha,25(OH)_2D_3$ (100 nM) for the indicated times. Proliferation of cells in $1\alpha,25(OH)_2D_3$- *versus* vehicle-treated cultures is shown.

We observed that *CST5* shRNA cells responded distinctly to 1α,25(OH)$_2$D$_3$ treatment that control shRNA cells. Thus, *CST5* shRNA cells showed an attenuated phenotypic change compared with control shRNA cells when they were treated with 1α,25(OH)$_2$D$_3$ (Figure 23B). In addition, when we analyzed the antiproliferative effect of 1α,25(OH)$_2$D$_3$ in these cells we found that it was abrogated by *CST5* knock-down (Figure 23C).

We also studied if *CST5* shRNA cells changed the pattern of gene expression. Conversely to the effect observed with cystatin D overexpression, in 1α,25(OH)$_2$D$_3$-treated *CST5* shRNA cells the induction of *CYP24* RNA was significantly lower (73%) than that in control shRNA cells (Figure 24A, left panel). Similar results were found for *CDH1*/E-cadherin RNA and protein expression (53% and 35% reduction, respectively) (Figure 24A, middle panel and 24B). The expression of the mesenchymal marker LEF-1 increased in *CST5* shRNA cells (1.2-fold) and the 1α,25(OH)$_2$D$_3$-mediated reduction of its RNA level was lower in these cells as compared to control shRNA cells (29% *versus* 42%) (Figure 24A, right panel and 24B). Moreover and in contrast with the effects of cystatin D overexpression, *CST5* knock-down increased c-MYC (2-fold) (Figure 24B) and SNAIL1 (1.5-fold) (Figure 24C) protein expression.

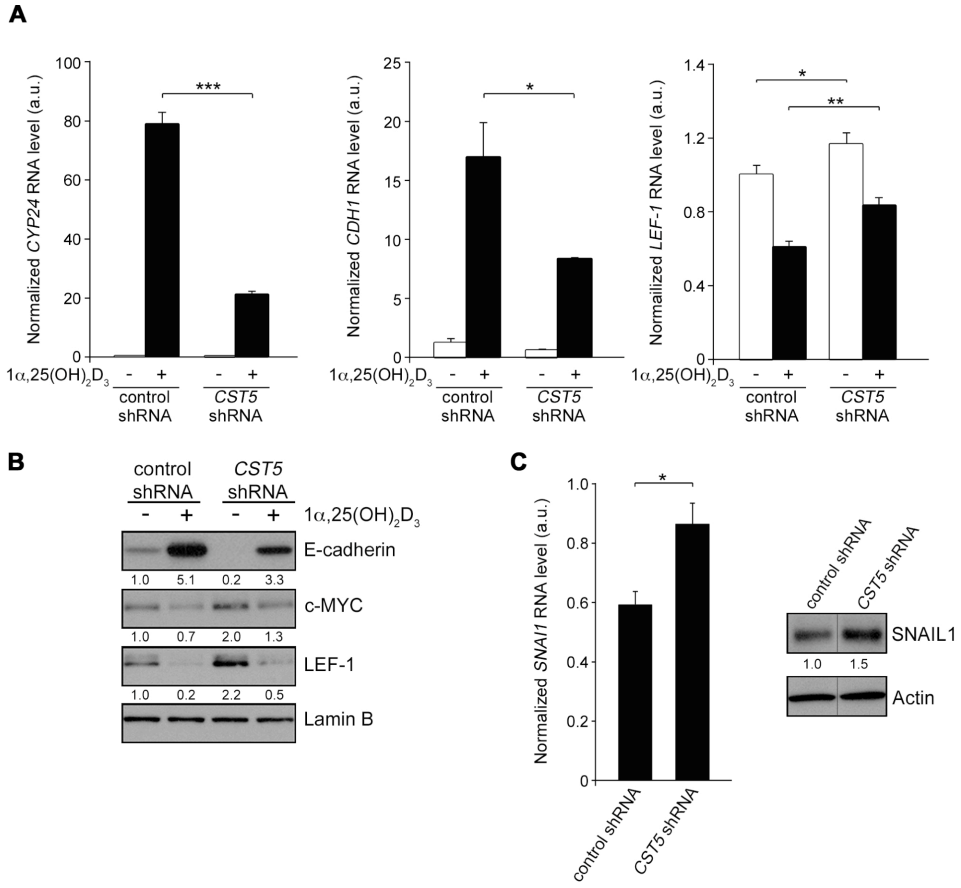

Figure 24. *CST5* knock-down affects gene expression in SW480-ADH cells. **(A)** Quantitative RT-PCR analysis showing decreased *CYP24* (left), *CDH1* (middle), and *LEF1* (right) RNA expression in *CST5* shRNA cells. **(B)** Western blot analysis showing changes in E-cadherin, c-MYC, and LEF-1 proteins in *CST5* shRNA cells. **(C)** *CST5* knock-down increases the expression of *SNAI1* RNA (left) and protein (right). Numbers between the blots correspond to mean of the fold change values obtained in two experiments.

Altogether, these results agree with those obtained by means of ectopic cystatin D expression, suggesting a role for this protease inhibitor mediating $1\alpha,25(OH)_2D_3$ antitumor activity.

6. Cystatin D inhibits proliferation, migration, and anchorage-independent growth of cultured colon cancer cells and their tumorigenic potential *in vivo*

To examine if cystatin D had any effect in other colon cancer cell lines, we expressed ectopically the human CST5 cDNA in LS174T, and HCT116 cells that have undetectable or low endogenous levels of this protease inhibitor. Expression of the exogenous cystatin D protein in transfected clones following antibiotic selection was analyzed by western blotting and two (#9 and #11) or four (#9, #17, #20, #28) clones (LS174T or HCT116, respectively) with different cystatin D expression levels were selected for further studies (Figure 25A and 25B). The activity of exogenous cystatin D was assessed in enzymatic assays (Figure 25C and 25D).

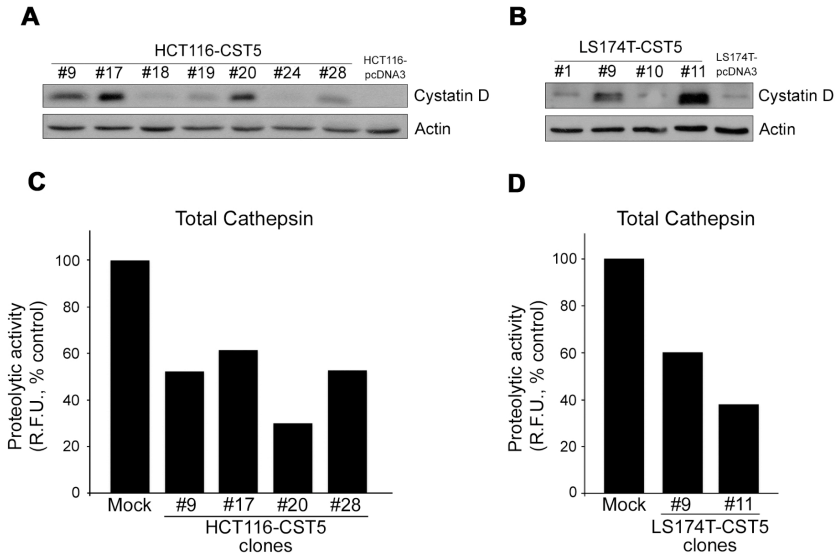

Figure 25. Ectopic cystatin D expression in stably-transfected HCT116 and LS174T cells. Western blot analysis showing the expression of cystatin D protein in G418-selected clones of transfected **(A)** HCT116 and **(B)** LS174T cells. Total cathepsin activity in selected **(C)** HCT116-CST5 and **(D)** LS174T-CST5 clones. Specific activity was expressed as relative fluorescence units (RFU).

Based on the results obtained in SW480-ADH cells we studied if exogenous cystatin D inhibited the proliferation of HCT116 and LS174T cells. Indeed, cystatin D overexpression drastically decreased the proliferation of both HCT116 (~ 85%) and LS174T (~ 54%) cell lines (Figure 26A and 26B). Conversely, neither recombinant cystatin D protein added to cultured medium nor the non-permeable E-64 pan-cathepsin inhibitor altered the proliferation

of these two cell lines, suggesting that this effect requires intracellular mechanisms (Figure 26C and 26D).

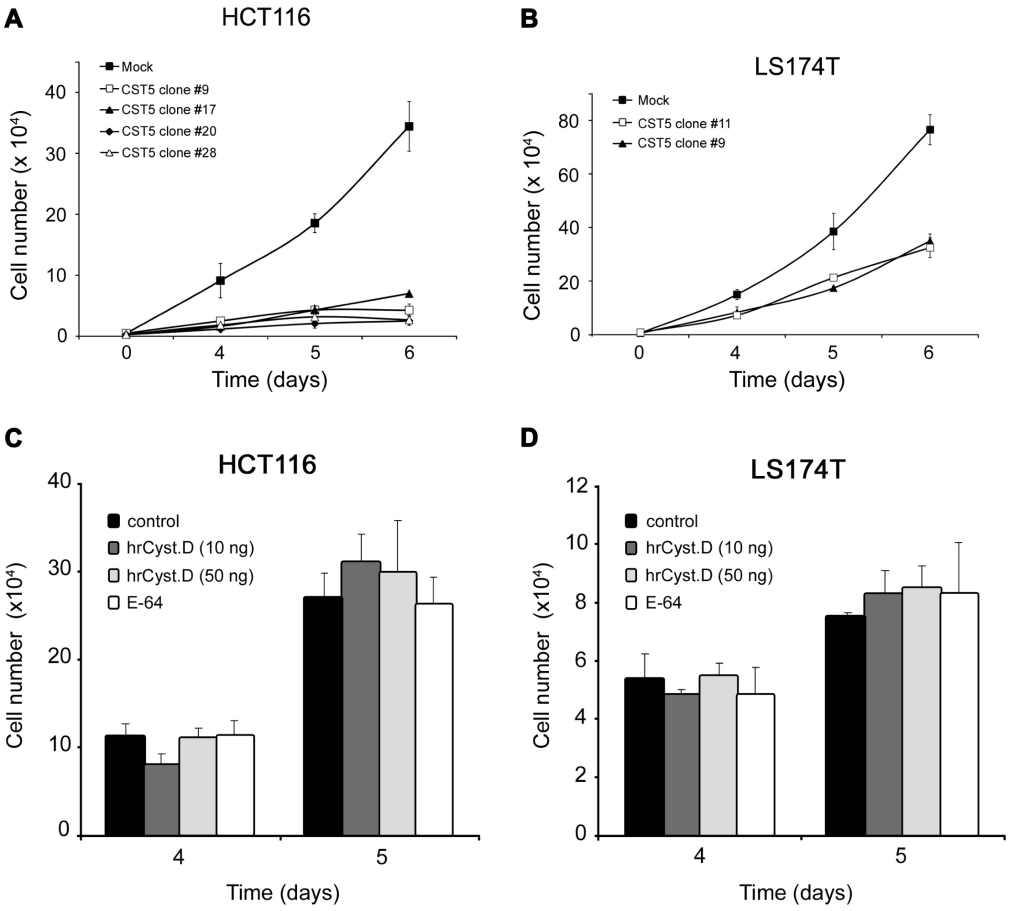

Figure 26. Ectopic cystatin D expression inhibits proliferation of colon cancer cells. Cystatin D inhibits cell proliferation of **(A)** HCT116 and **(B)** LS174T cells. HCT116 or LS174T cells transfected with empty vector (Mock) or stably expressing CST5 were seeded in 24-well plates and after 6 days living cells were counted after trypsinization. Representative data from three independent experiments done in triplicate are shown. **(C)** HCT116 and **(D)** LS174T cell proliferation is unaffected by human recombinant cystatin D (hrCyst.D) protein or the non-permeable E-64 pan-cathepsin inhibitor. Eight thousand cells were seeded and incubated for the indicated times in growth medium containing hrCyst.D, E-64 or vehicle.

We also studied two *in vitro* parameters of cell transformation: migration and anchorage-independent growth. In these experiments we used HCT116 cells since LS174T cells lack basal migration capacity. Cystatin D expression significantly ($P < 0.001$) reduced migration of HCT116 cells in Boyden chamber assays (Figure 27A). Likewise, cystatin D inhibited the growth of HCT116 cells in semisolid agar ($P < 0.001$), and the colonies formed by cystatin D-expressing HCT116 cells had also a smaller size than that of control cells (Figure 27B).

Given that exogenous cystatin D inhibits several parameters of malignancy *in vitro* we examined the antitumor effects of cystatin D *in vivo*. To this end, we injected subcutaneously cystatin D-expressing or control HCT116 or LS174T cells in immunodeficient mice. The volume of the tumors was measured every two days and the mice were sacrificed at day 23 and 24 post-injection, respectively (Figure 28). In both cases, the number of tumors generated was drastically inhibited by cystatin D expression, as at the end of the experiment

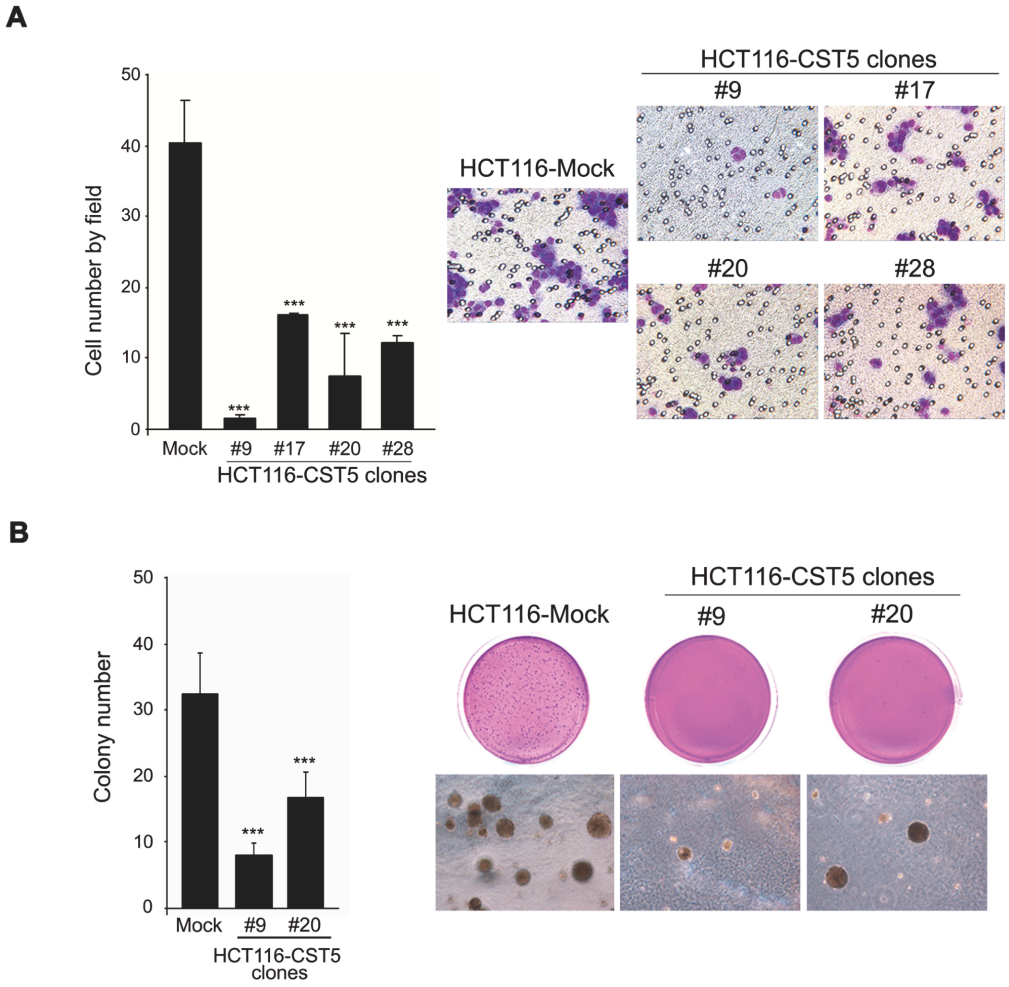

Figure 27. Ectopic cystatin D expression inhibits migration and anchorage-independent growth of HCT116 cells. **(A)** Cystatin D inhibits cell migration. HCT116 cells transfected with empty vector (Mock) or stably expressing CST5 were seeded in triplicate on Transwell® filters, and 24 h later migratory cells that had attached to the lower surface of filters were counted. Representative images and a quantification of data from three independent experiments are shown. **(B)** Cystatin D inhibits anchorage-independent cell growth. Images of *foci* grown in semisolid agar and quantification are shown. Original magnification, x63.

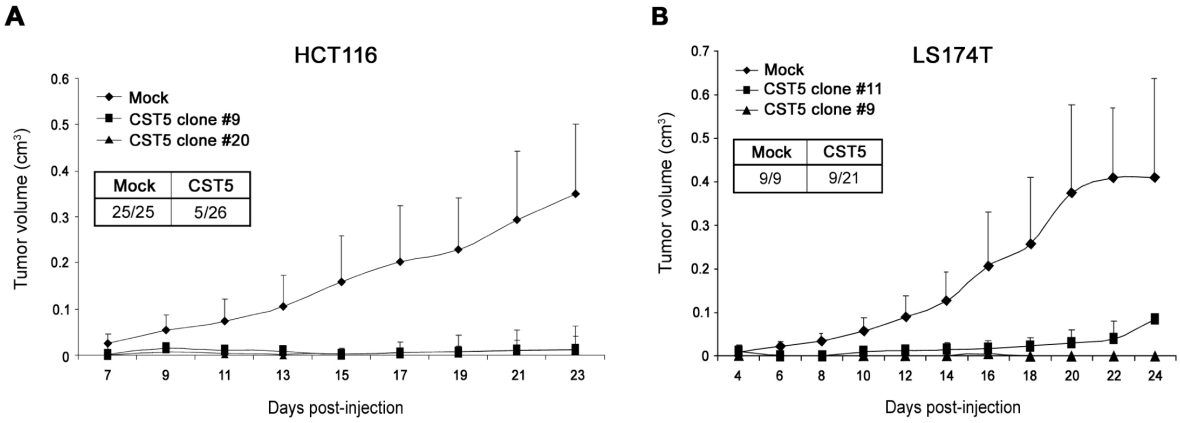

Figure 28. Cystatin D inhibits tumor growth *in vivo*. Immunodeficient mice were injected subcutaneously with **(A)** mock-transfected HCT116 cells or one of the two clones of HCT116 cells transfected with CST5 (clone #9 or #20); or **(B)** mock-transfected LS174T cells or one of the two clones of LS174T cells transfected with CST5 (clone #9 or #11). The volumen of the tumors generated was measured during the indicated period. Inset, number of mice developing tumors at the end of the evaluating period.

100% of mice injected with control HCT116 or LS174T cells had developed tumors whereas this decreased to only 19% and 43% of mice injected with cystatin D-expressing HCT116 or LS174T cells, respectively. Tumor volume was also significantly decreased ($P < 0.001$) in both cystatin D-expressing cells.

These results show that cystatin D has antitumor activity in colon cancer cell lines independently of $1\alpha,25(OH)_2D_3$.

7. Cystatin D induces intercellular adhesion proteins and inhibits genes promoting epithelial-mesenchymal transition

Cells expressing exogenous cystatin D exhibited, when they were in culture, a strong adhesive phenotype (Figure 29, left panels). Therefore, we analyzed the expression of adhesion proteins in these cells. Immunofluorescence and confocal microscopy analyses showed that cystatin D-expressing HCT116 cells had increased level of the *adherens junction* E-cadherin and p120-catenin and of the tight junction occludin proteins (Figure 29, right panels). This result was also confirmed by western blot analysis (Figure 30A).

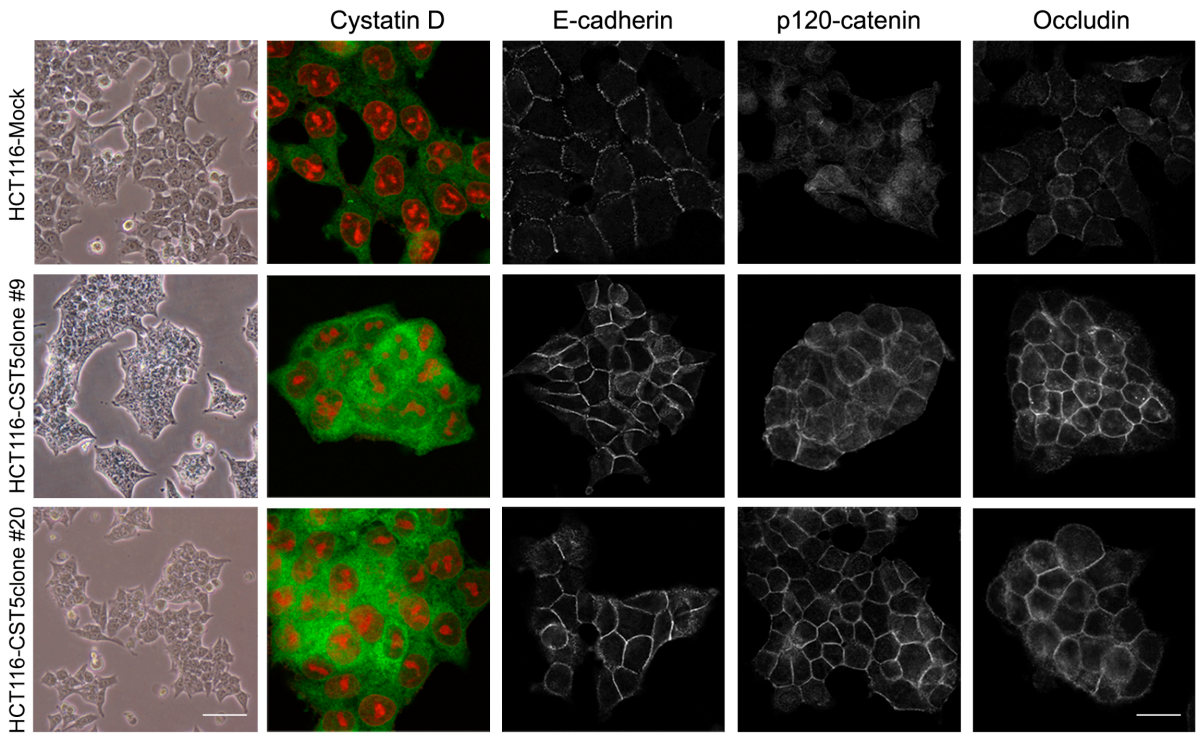

Figure 29. Ectopic cystatin D induces intercellular adhesion. Phase-contrast (left) and confocal microscope images (right) of control (mock) and cystatin D-expresing HCT116 cells. Expression of E-cadherin, p120-catenin, and occludin proteins was analyzed using specific antibodies. Scale bars: 10 μm (left) and 20 μm (right).

We selected E-cadherin for a more detailed study of its regulation by cystatin D. First, we corroborated that cystatin D-expressing cells contained higher level of *CDH1* RNA than control cells (Figure 30B). Moreover, when we assayed the activation of a *CDH1* promoter fragment (-987/+92) we found that it was more activated in cystatin D-expressing cells than in control cells (Figure 30C). As E-cadherin and occludin are targets of transcriptional inhibition by genes promoting EMT, we studied whether cystatin D could modulate these genes. Using quantitative RT-PCR assays we observed that indeed, cystatin D-expressing cells expressed lower level of *SNAI2, ZEB1* and *ZEB2* RNA than mock cells (Figure 31). In contrast, only a weak reduction of *SNAI1* and no change of *TWIST* expression were found.

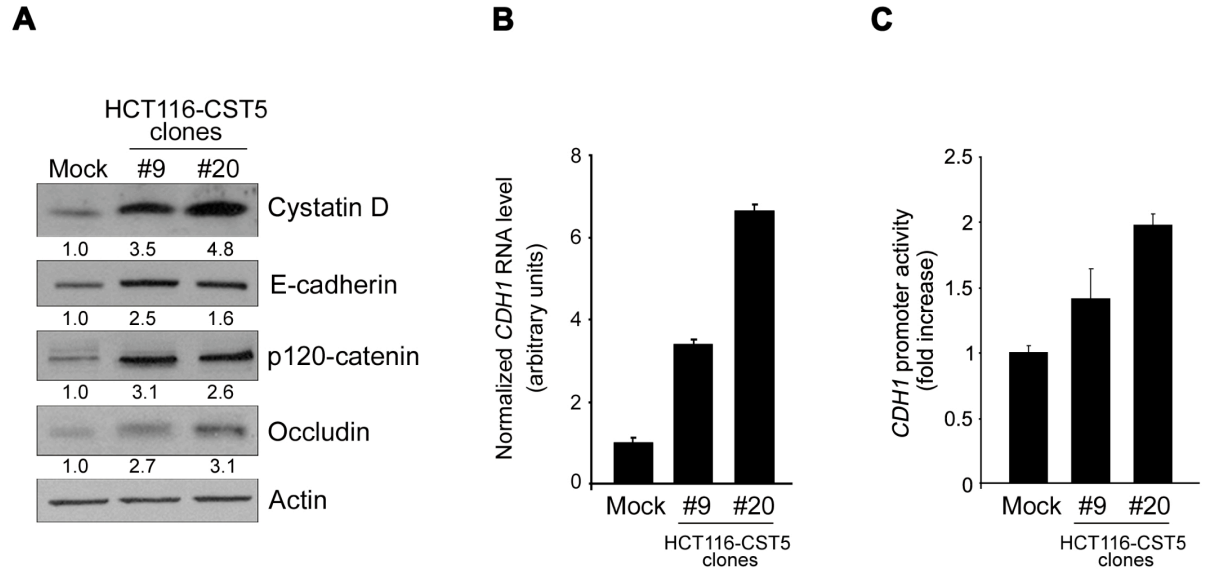

Figure 30. Ectopic cystatin D regulates E-cadherin expression. **(A)** Western blot analysis showing the induction of E-cadherin and other adhesion proteins by cystatin D in HCT116 cells. Numbers between the blots correspond to mean of the fold change values obtained in three experiments. **(B)** Quantitative RT-PCR analysis showing increased levels of *CDH1* RNA in cystatin D-expressing HCT116 cells. **(C)** Effect of ectopic cystatin D on *CDH1* promoter in HCT116 cells. The cells were transfected with a *CDH1* promoter fragment, and luciferase activity was measured 48 h later.

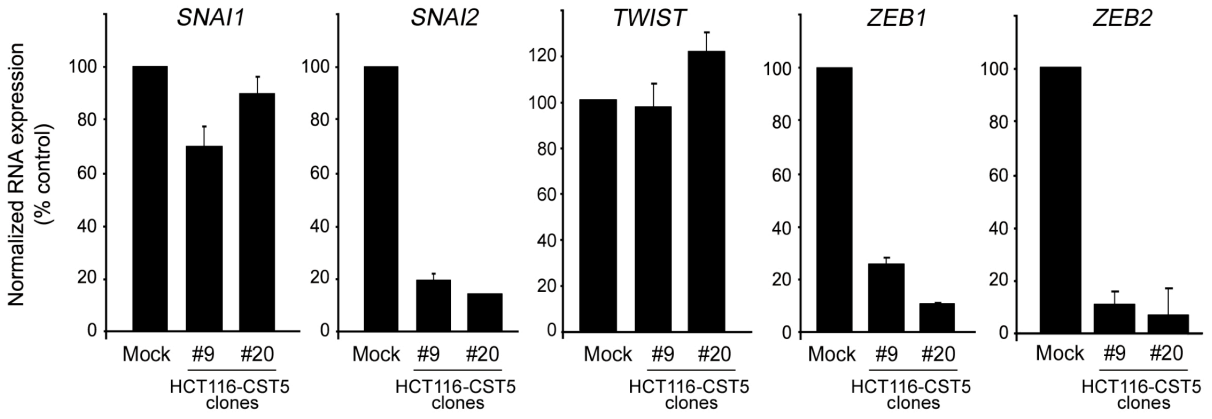

Figure 31. Cystatin D represses genes involved in EMT. Quantitative RT-PCR analysis of the expression of the EMT genes *SNAI1, SNAI2, TWIST, ZEB1* and *ZEB2* in mock and cystatin D-expressing HCT116 cells.

Altogether these results show that cystatin D induces an adhesive phenotype in colon cancer cells mediated at least in part by the induction of adhesion proteins and the downregulation of their repressors.

8. Cystatin D extends the cell cycle and inhibits β-catenin/TCF transcriptional activity and the *c-MYC* oncogene

To explore the mechanism by which cystatin D inhibits cell proliferation we first performed flow cytometry analyses. Cells were synchronized using thymidine and L-mimosine, increasing thus the proportion of G_1-arrested cells. Cystatin D-expressing cells displayed slower entry into cell cycle upon syncronization than control cells, as shown by the lower proportion of cells in G_2/M phase at 4 hours after release of the cell cycle blockade (Figure 32). This explains their lower proliferation rate.

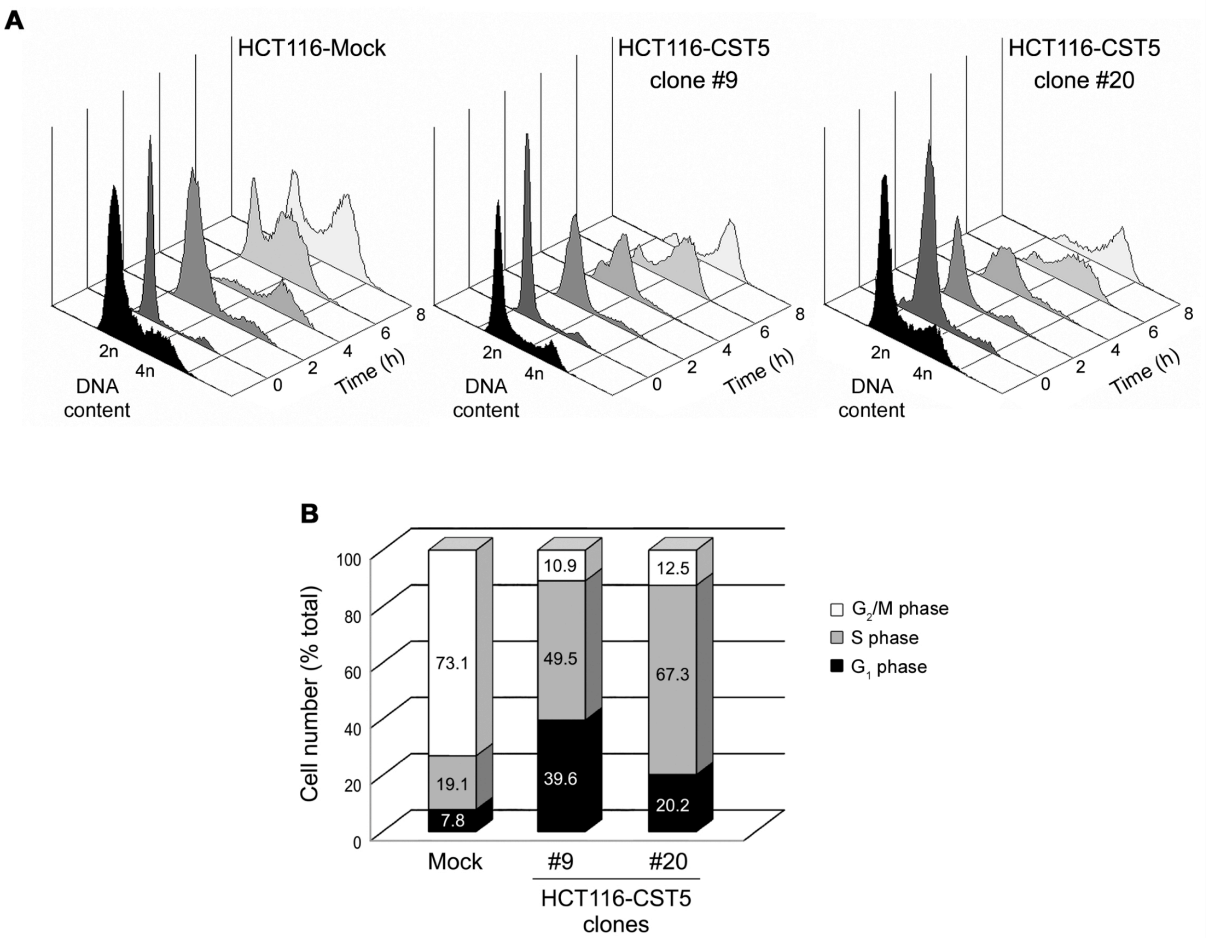

Figure 32. Cystatin D extends the cell cycle. (A) Cystatin D decelerates cell-cycle entry. Mock and cystatin D-expressing HCT116 cell clones were stimulated with 10% serum to enter into cell cycle following synchronization in G_1 phase using double block with thymidine and mimosine as described in Methods, and the proportion of cells in each phase was estimated by flow citometry. Asynchronously growing cells were used for comparison (black profile). Data from a representative experiment of three performed are shown. **(B)** Graph representing the mean percentage of cells present in each phase of the cell cycle 4 h after release of the blockade.

We also analyzed c-MYC protein expression. Western blot analysis revealed that cystatin D-expressing cells had a decreased level of c-MYC protein (Figure 33A). Consistently, two *c-MYC* promoter constructs were less active in cystatin D-expressing cells than in control cells (Figure 33B). As the aberrant activation of the Wnt/β-catenin pathway that causes the induction of *c-MYC* and other proliferation and invasion genes is a hallmark of colon cancer [47,267], we examined its activity in cystatin D-expressing cells. Analogously to $1\alpha,25(OH)_2D_3$ treatment [225,269], exogenous cystatin D inhibited the transcriptional activity of β-catenin/TCF complexes, the downstream effector of the Wnt pathway (Figure 33C). This does not result from a general inhibitory effect on transcription as the Notch pathway, evaluated by using a Notch intracellular domain (NICD) reporter construct, was not affected by cystatin D expression (Figure 33D).

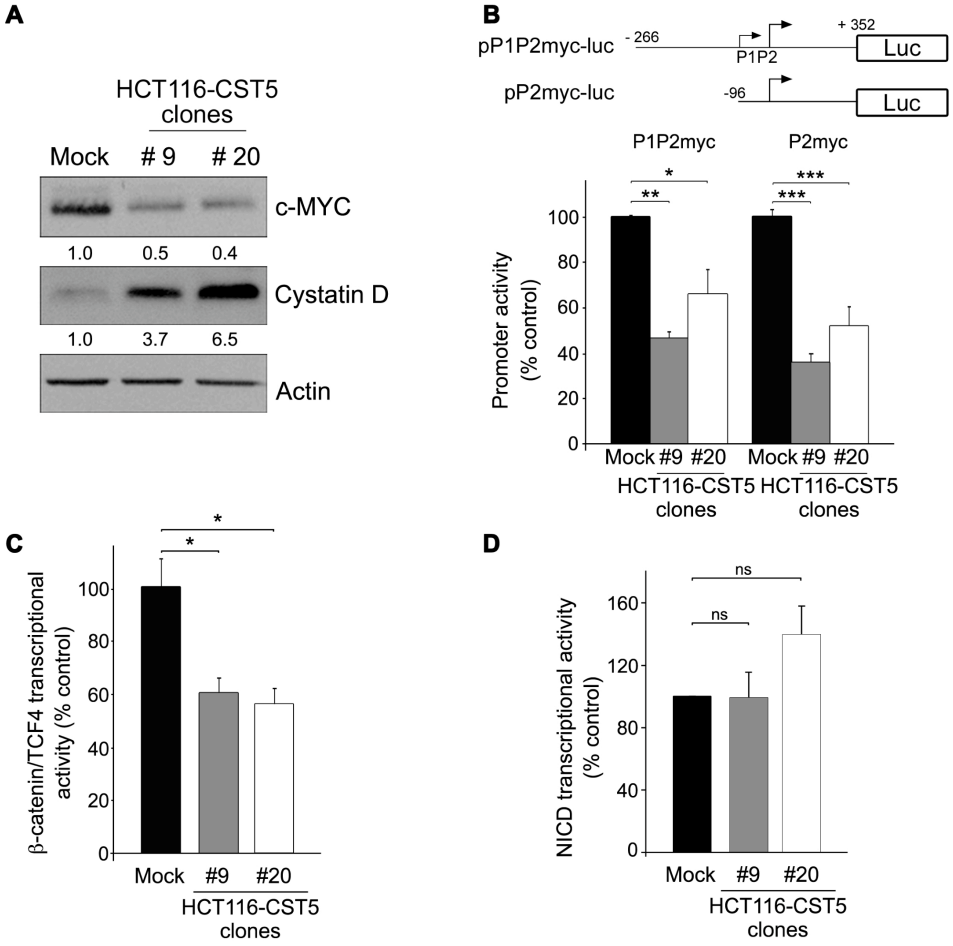

Figure 33. Cystatin D inhibits c-MYC expression and β-catenin/TCF transcriptional activity. **(A)** Western blot analysis showing the decrease in c-MYC protein content in cells expressing exogenous cystatin D following the release of cell-cycle blockade. Numbers between the blots correspond to mean of the fold change values obtained in three independent experiments. **(B)** The human *c-MYC* gene promoter is less active in cystatin D-expressing cells. Mock and two cystatin D-expressing HCT116 cell clones were transfected with either of two constructs of the *c-MYC* promoter, and luciferase activity was measured 48 h later. **(C)** β-catenin/TCF transcriptional activity is reduced in cystatin D-expressing cells. Mock and two cystatin D-expressing HCT116 cell clones were transfected with the wild-type TOP-Flash and mutant FOP-Flash reporter plasmids, and the TOP/FOP ratio of luciferase activity was measured 48 h later. **(D)** Notch function was measured by the degree of CBF1 binding using the wild-type 4xCBF1wt-Luc compared with background binding to a mutant 4xCBF1mut-Luc. The 4xCBF1wt/4xCBF1mut ratio was measured 48 h later.

We conclude that the antiproliferative action of cystatin D is at least in part mediated by the repression of the *c-MYC* oncogene, which in turn is probably mediated partially by the inhibitory effect on the Wnt/β-catenin pathway.

9. Cystatin D proteins with reduced antiproteolytic activity maintain the antiproliferative but not the migration inhibitory effect

To examine whether the antiproteolytic activity of cystatin D is necessary for its newly identified effects on cancer cells, we generated by PCR-mediated mutagenesis two mutant forms of this protease inhibitor (Figure 34A). One of these cystatin D mutants had a Gly replacing Trp at position 108 (CST5 W108G). This Trp has been described as a crucial mediator of cystatin C antiproteolytic capacity (W106 in cystatin C), and its mutation (W106G) in this cystatin caused a large decrease in target enzyme affinity [107]. Since cystatin D has great homology with cystatin C, and although it is known that the region where W108 is located (loop L2) has a lesser contribution in cystatin D affinity than in cystatin C [5], we considered that this mutation would be of interest. We also generated a double mutant that carried this mutation and in addition a deletion of the first 12 amino acids (CST5 W108G/Δ1-12). As it has been already reported, a mutant cystatin D that lacks the first 11 aminoacids (N-terminal segment) is mostly inactive as an inhibitor [106].

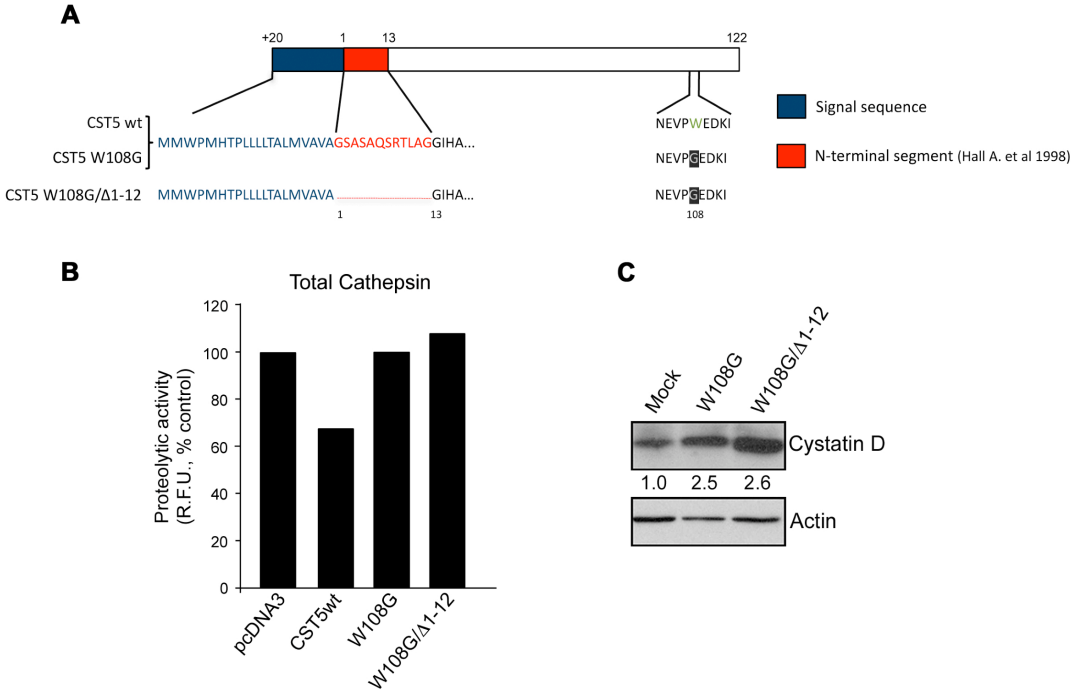

Figure 34. Generation of cystatin D mutant proteins with reduced antiproteolytic activity. **(A)** Schematic representation of the two cystatin D mutant proteins. CST5 W108G harbors a point Trp to Gly mutation at position 108 (W108G, highlighted by a black square), while CST5 W108G/Δ1-12 harbors the same W108G mutation and a deletion of the first twelve N-terminal aminoacids (in red). **(B)** Total cathepsin activity in COS-7 cells transfected with the indicated cystatin D cDNAs or with empty pcDNA3 vector. **(C)** Western blot analysis showing the expression of CST5 W108D and CST5 W108G/Δ1-12 proteins in HCT116 cells. Numbers between the blots correspond to mean of the fold change values obtained in three independent experiments.

Mutations were verified by sequencing, and the loss of cysteine protease activity was checked by transfecting the mutant CST5 constructs in COS-7 cells (Figure 34B). To evaluate if mutant cystatin D proteins retained the previously reported antitumor activity, we expressed them in HCT116 cells and verified their expression by western blot (Figure 34C).

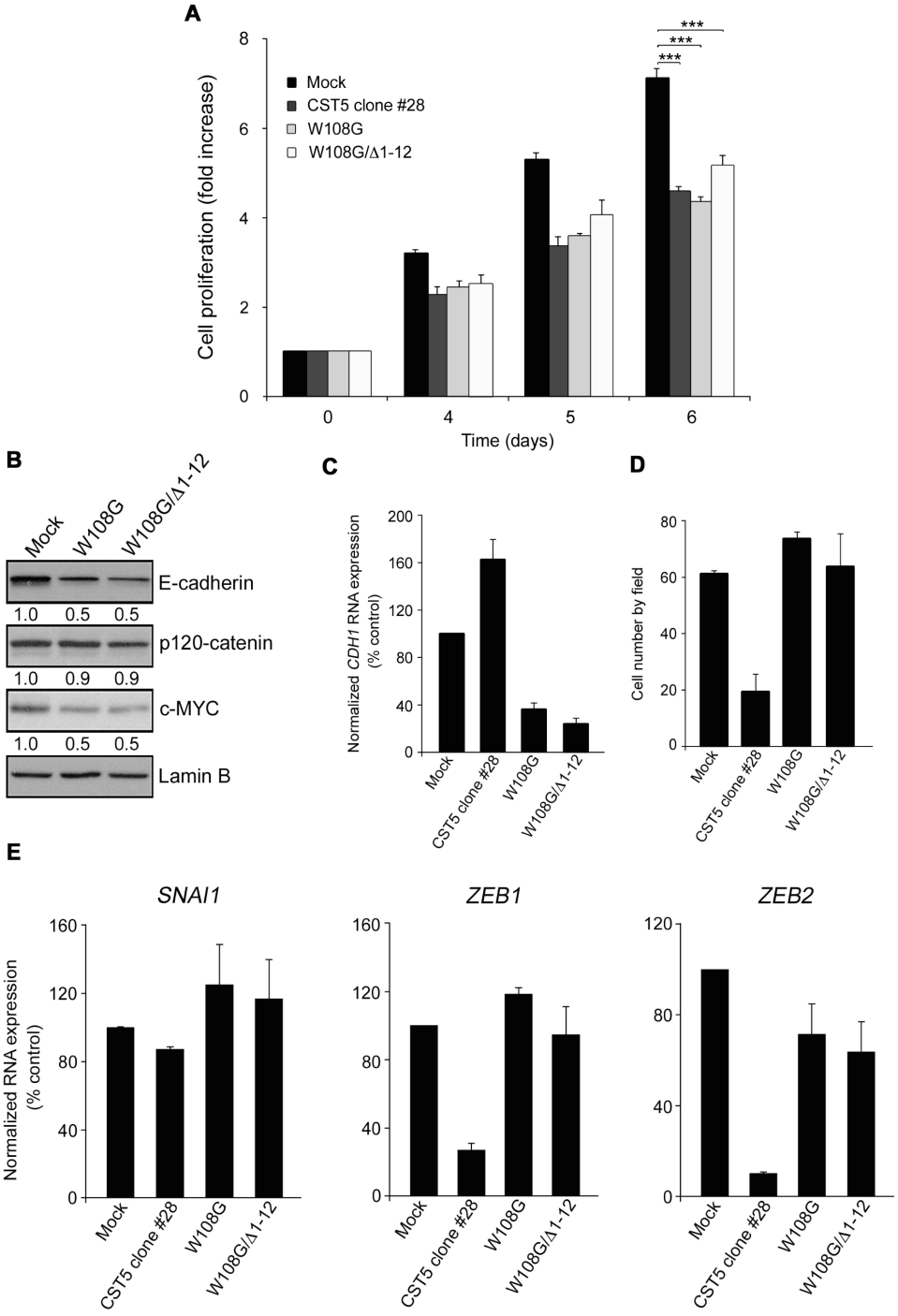

Figure 35. Mutant cystatin D proteins distinctly affect cell proliferation, migration and gene expression. **(A)** CST5 W108G and CST5 W108G/Δ1-12 have the same antiproliferative effect as wild-type cystatin D. **(B)** Western blot analysis showing E-cadherin, p120-catenin, and c-MYC protein expression in cells expressing mutant cystatin D proteins. Numbers between the blots correspond to mean of the fold change values obtained in three independent experiments. **(C)** Quantitative RT-PCR analysis showing the RNA levels of *CDH1* in cells expressing either wild-type or mutant cystatin D proteins. **(D)** CST5 W108G and CST5 W108G/Δ1-12 lack migration-inhibitory activity. **(E)** Quantitative RT-PCR analysis showing the RNA levels of *SNAI1, ZEB1,* and *ZEB2* in cells expressing either wild-type or mutant cystatin D proteins.

Next, we studied their effects on cell proliferation. Similar to the results obtained with wild-type cystatin D, both mutant proteins inhibited cell proliferation (Figure 35A). In view of this result, we analyzed c-MYC protein expression, and in line with their antiproliferative effect we observed that mutant proteins decreased the level of this protein, similarly to wild-type cystatin D (Figure 35B).

However, when we analyzed other parameters affected by wild-type cystatin D expression, as the migration capacity of the cells, we observed that in contrast to the previous findings with wild-type protein, none of the two mutant cystatin D decreased cell migration (Figure 35D). In addition, cells expressing either of the mutant cystatin D proteins did not show an adhesive phenotype like wild-type cystatin D-expressing cells. Therefore, we studied the effect of both cystatin D mutants on adhesion proteins. As it can be seen in Figure 35B, and also in contrast to wild-type cystatin D, the two mutant proteins decreased E-cadherin, while they did not alter p120-catenin expression. The decreased expression of E-cadherin was also studied at the RNA level, with similar results as that observed at the protein level (Figure 35C). Consistently, when we studied the expression of EMT genes (Figure 35E), the two mutant cystatin D proteins failed to inhibit *ZEB1* RNA expression and were less efficient than wild-type cystatin D to repress *ZEB2*. Moreover, they slightly increased the expression of *SNAI1* RNA.

Altogether, these results indicate that the inhibition of cysteine proteases is only partially responsible for the effects of cystatin D in colon cancer cells.

10. Cystatin D expression decreases in human colorectal tumorigenesis in good correlation with tumor dedifferentiation and the loss of VDR and E-cadherin expression

Previous results show the effects of cystatin D in colon cancer cell lines. We wished to analyze the relevance of our results on human colon cancer. To this purpose, we first studied the expression of cystatin D protein in tissue microarrays containing tumor and adjacent normal tissue samples. This study was performed in collaboration with Dr. Aurora Astudillo's group from Servicio de Anatomía Patológica, IUOPA-HUCA, Oviedo.

By inmunohistochemical analysis we studied samples with diverse differentiation grade: non-tumor tissue (n = 7), well-differentiated (n = 7), moderately-differentiated (n = 24) and poorly-differentiated (n = 13) tumors. We found a progressive loss of cystatin D expression that correlated with tumor dedifferentiation: while in normal tissue, polyps, and well-differentiated carcinomas cystatin D showed a strong staining, its expression decreased significantly in moderately-differentiated carcinomas (12.5% showed negative staining and no samples presented a very high level of staining) and was absent in a high proportion of

poorly-differentiated tumors (38.5%, and no samples had high or very high staining level). In line with previous studies [236] a correlation between downregulation of VDR and poor differentiation of colorectal tumors was found (Figure 36).

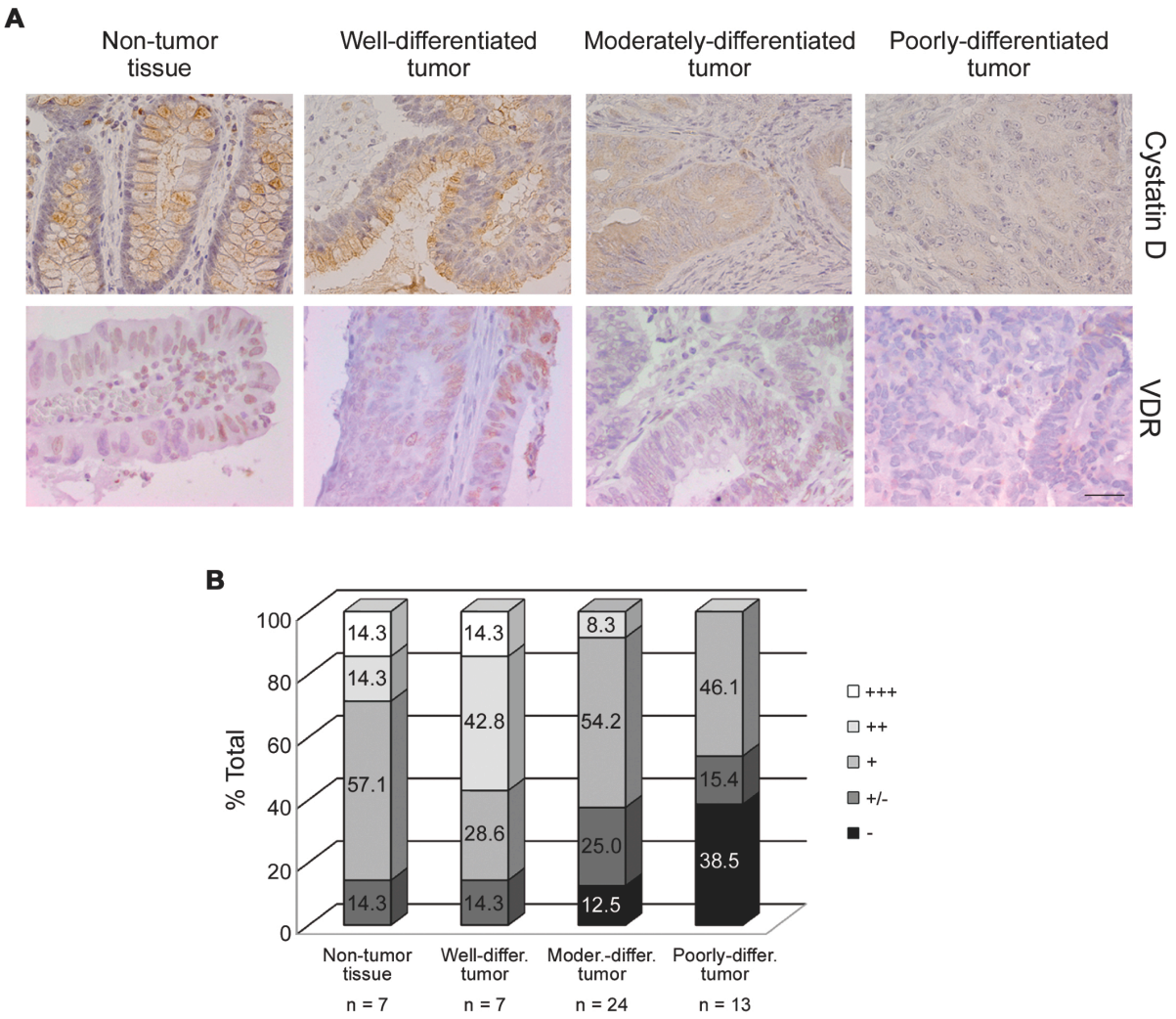

Figure 36. Cystatin D expression is downregulated during human colon cancer progression. (A) Immunohistochemical analysis of cystatin D and VDR expression in tissue microarrays. Counterstaining was with hematoxylin. Representative slices of non-tumor tissue and of well-, moderately-, and poorly- differentiated carcinomas. Scale bar: 200 μm. **(B)** Quantification of cystatin D expression by estimation of staining intensity as described in Methods. The number of samples analyzed *per* group and the percentage corresponding to each level of cystatin D staining are shown.

These results were confirmed by western blot analysis. We analyzed cystatin D expression in colonic normal and tumor tissue samples from 32 CRC patients. We also analyzed the expression of VDR and E-cadherin due to their known role as markers of differentiation of human colorectal tumors [236]. Results are expressed as tumor *versus* normal (T/N) ratio of normalized protein levels of cystatin D, VDR and E-cadherin. We defined overexpression and reduction as changes of at least 2-fold in protein level. In 40% (13/32) cases, the level of cystatin D was lower in tumor than in normal tissue, in 44% (14/32) was equal in tumor and

normal tissue and only in 15% (5/32) the level of cystatin D was greater in tumor than in normal tissue (Figure 37).

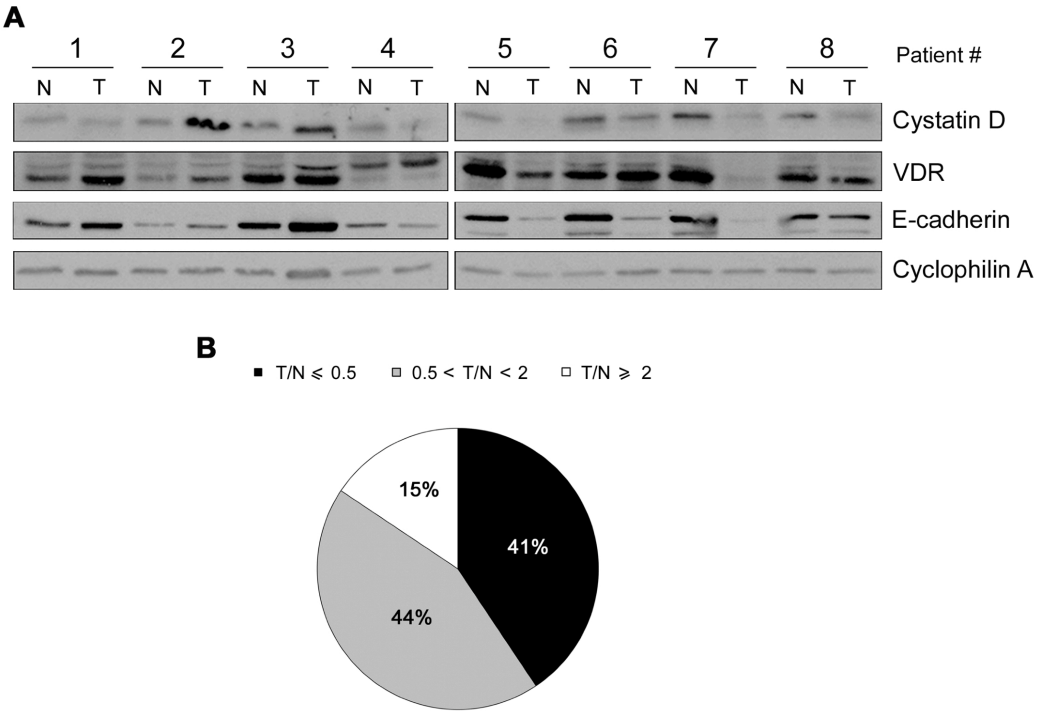

Figure 37. Cystatin D expression in colon carcinomas. **(A)** Representative western blot showing the expression of cystatin D, VDR, and E-cadherin proteins in a series of matched normal (N) and tumor (T) human colon tissues. **(B)** Percentage of patients in each group of normalized cystatin D protein expression. CRC patients were classified in three subgroups: less expression in tumor than in normal tissue (T/N ≤ 0.5), equal expression in tumor and normal tissue (0.5 < T/N < 2.0) or more expression in tumor than in normal tissue (T/N ≥ 2.0).

Additionally, we represented the results in two scattergrams (Figure 38A) where one axis corresponds with the expression [as $\log_2$ (T/N)] of VDR or E-cadherin, and the other one with that of cystatin D. We found a strong direct correlation between the expression of cystatin D and VDR (Spearman correlation coefficient r = 0.562; *P* = 0.001) supporting that the regulation of cystatin D expression by $1\alpha,25(OH)_2D_3$ observed in cultured cells and xenografts may also take place in human colon cancer. Moreover, the expression of cystatin D also correlated with that of the E-cadherin (Spearman correlation coefficient r = 0.492; *P* = 0.005).

Subsequently, we analyzed the data considering cystatin D expression as a qualitative variable and we divided the patients in two groups according with high (50% of samples) or low (50% of samples) cystatin D expression. Box-plot analysis of VDR and E-cadherin expression regarding to that of cystatin D revealed that both VDR and E-cadherin expression was significantly greater in tumors with high cystatin D expression than that with low one (Kruskal-Wallis test *P* = 0.001 and *P* = 0.006, respectively) (Figure 38B).

From these results we conclude that cystatin D expression directly correlates with that of VDR and E-cadherin in colon carcinomas both associated with tumor dedifferentiation.

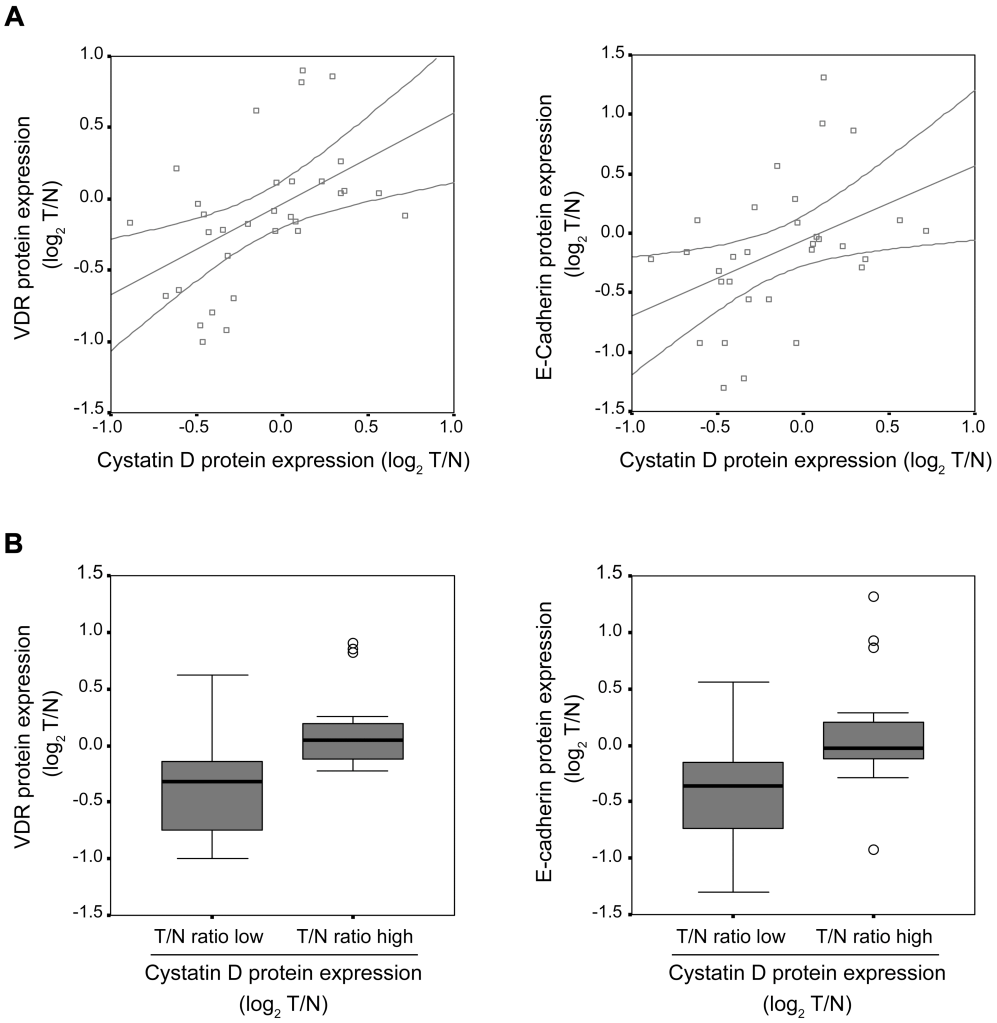

Figure 38. Cystatin D expression directly correlates with that of VDR and E-cadherin in colon carcinomas. **(A)** Scattergram showing the relationship between the $\log_2$ tumor *versus* normal (T/N) ratio of normalized protein levels of cystatin D and VDR (left) or E-cadherin (right). **(B)** Box-plot of the $\log_2$ T/N ratio of normalized VDR (left) or E-cadherin (right) expression in samples with high or low cystatin D levels in the colorectal cancer series. Boxes include values in the 25%-75% interval; internal lines represent the median; the outliers (circles) of the VDR and E-cadherin expression are indicated.

11. Gene expression profile induced by cystatin D

In view of the newly identified effects of cystatin D in colon cancer cells, and considering the evidence of cathepsin inhibition-independent mechanisms involved in them, we analyzed using oligonucleotide microarrays changes in gene expression related with cystatin D overexpression in HCT116 cells (MIN phenotype; wid-type *TP53* and *APC*, mutated *K-RAS, CTNNB1*/β-catenin*, PI3KCA* and *TGFβRII*). For these assays we used two different clones of cells expressing exogenous cystatin D (#9 and #20) as well as mock and wild-type cells. The two clones were used as biological replicates (HCT116 CST5), and the same criterion was

assumed to mock and wild-type cells (HCT116 Control). We considered cystatin D-regulated genes those with at least 1.5-fold difference in expression as compared to Control cells. This analysis revealed that cystatin D overexpression modifies the expression of 69 genes: 23 genes were upregulated (33%) whereas 46 genes were downregulated (67%).

These genes were classified according to their function. Figure 39 shows the most represented categories and the percentage of genes in each one. The categories with a greater number of genes were cell adhesion, cytoskeleton and extracellular matrix, followed by transcription and signal transduction.

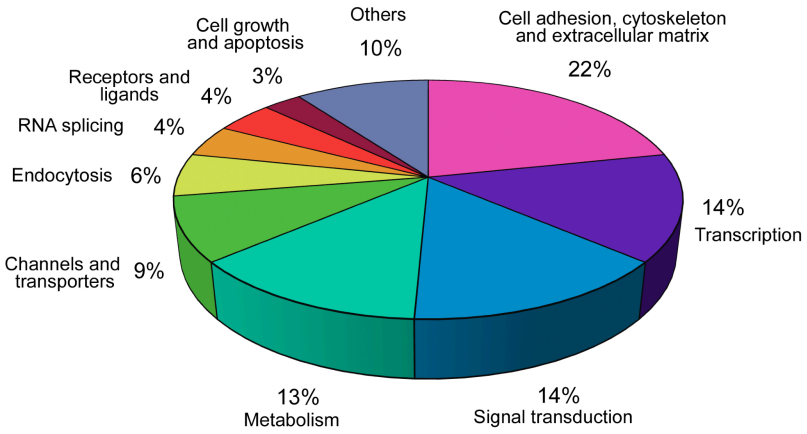

Figure 39. Functional distribution of candidate target genes regulated by cystatin D overexpression in HCT116 cells. The most represented categories and the percentage of genes included in each one are shown.

Table VII shows the genes included in each category. We found genes such as *CDH1* or *CTNND1* whose regulation by exogenous cystatin D was already showed in this work. However, *CTNND1*, which encodes p120-catenin, is not present in the table since its value (1.19) does not achieve the criterion used in the analysis.

Exogenous cystatin D increased the expression of important genes related with cell adhesion such as *CDH1* or *CLDN7*, which encode proteins of *adherens* and tight junctions, respectively, or *JUP* (1.46) that encodes desmoplakin-3, a desmosomal protein. In addition, exogenous cystatin D inhibited the expression of other cell adhesion and cytoskeleton related genes such as *VCAN*, *MSN* or *VIM*, which have been related with cancer progression [43,152,257,271,329].

Several transcription factors such as *MEF2C*, *SOX4*, *RUNX2* or *ID3* were induced, whereas *NPAS2*, *RUNX1* or *ELK3* were inhibited by exogenous cystatin D. Notably, all genes related with metabolism regulated by cystatin D in microarray data were inhibited.

We validated various genes regulated by cystatin D in the microarray study analyzing by quantitative RT-PCR their expression in cystatin D-expressing HCT116 clones #9 and #20.

This study confirmed the regulation of 12/13 genes (considering regulation a $2^{-\Delta\Delta C}{}_T \geq 2.00$ for induced genes and $2^{-\Delta\Delta C}{}_T \leq 0.50$ for inhibited ones, calculated as described in Materials and Methods). As control we used two endogenous genes (*18S* and *GADPH*) and similar results were observed (Figure 40).

Table VII. Genes regulated by cystatin D overexpression in HCT116 cells

Gene Symbol	Title	RNA level
TRANSCRIPTION		
MEF2C	Myocyte-specific enhancer factor 2C	4.26
AP1M2	Adaptor-related protein complex mu-2 subunit	3.77
SOX4	Transcription factor SOX (SRY-related HMG-box) 4	2.40
RUNX2	Runt-related transcription factor 2	2.17
ID3	Inhibitor of DNA binding 3	2.14
JARID1B	Histone demethylase Jumonji/ARID domain-containing protein 1B	1.73
ELK3	ETS domain-containing protein Elk-3	0.65
RUNX1	Runt-related transcription factor 1	0.58
NPAS2	Neuronal PAS domain-containing protein 2	0.51
TESC	Tescalcin	0.47
CELL ADHESION, CYTOSKELETON AND EXTRACELLULAR MATRIX		
CDH1	E-cadherin	3.63
KRT23	Keratin type I cytoskeletal 23	2.00
CLDN7	Claudin 7	1.91
IGSF9	Immunoglobulin superfamily member 9	1.80
MYO10	Myosin X	1.55
LAMC1	Laminin subunit gamma-1 precursor	0.65
STEAP1	Metalloreductase STEAP1 (six-transmembrane epithelial antigen of prostate 1)	0.61
MSN	Moesin	0.58
EMP3	Epithelial membrane protein 3	0.56
TSPAN5	Tetraspanin 5	0.54
COL12A1	Collagen alpha-1 (XII) chain precursor	0.53
FRAS1	Extracelullar matrix protein FRAS1 precursor	0.53
VIM	Vimentin	0.42
CEP170	Centrosomal protein of 170 kDa	0.35
VCAN	Versican core protein precursor	0.25
METABOLISM		
TXN	Thioredoxin	0.66
AKR1C2	Aldo-keto reductase family 1 member C2	0.65
DDT	D-dopachrome decarboxylase	0.64
NT5DC3	5'-nucleotidase domain containing 3 isoform 1	0.60
CYBRD1	Cytochrome b reductase 1	0.59
PYGB	Glycogen phosphorylase brain form	0.58
GDA	Guanine deaminase	0.57
GALNT5	Polypeptide N-acetylgalactosaminyltransferase 5	0.47
NT5E	5'-nucleotidase precursor (CD73 antigen)	0.20
SIGNAL TRANSDUCTION		
SYT7	Synaptotagmin 7	1.53
GRAP1	GRB2-related adapter protein	1.51
ARHGAP12	Rho-GTPase-activating protein 12	0.59
PDCL3	Phosducin-like protein 3	0.57
AKAP12	A-kinase anchor protein 12	0.57
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein	0.52
ARHGAP29	PTPL1-associated RhoGAP 1	0.42
ANXA3	Annexin A3	0.40
VSNL1	Visinin-like protein 1	0.23
NAV3	Neuron navigator 3	0.17

Table follows on the next page

Gene Symbol	Title	RNA level
CHANNELS AND TRANSPORTER		
SLC1A3	Excitatory amino acid transporter 1	2.51
ATP8B1	Probable phospholipid-transporting ATPase 1C	2.43
ATP2C2	Calcium-transporting ATPase type 2C member 2	1.77
TMC4	Transmembrane channel-like protein 4	1.59
RBP1	Cellular retinol-binding protein	0.62
SLC7A11	Cystine/glutamate transporter	0.60
ENDOCYTOSIS		
MAL2	MAL 2	8.03
EPN3	Epsin 3	1.58
VTI1B	Vesicle transport v-SNARE protein Vti-1 like 1	0.65
SNX30	Sortin nexin 30	0.62
RECEPTORS AND LIGANDS		
NR3C1	Glucocorticoid receptor	0.55
NRP1	Neuropilin 1 precursor	0.53
WNT16	Protein Wnt 16 precursor	0.36
RNA SPLICING		
RBM47	RNA-binding protein 47	1.77
RBM35A	RNA-binding protein 35A	1.62
RNPC3	RNA-binding region containing 3	0.56
CELL GROWTH AND APOPTOSIS		
PPP2R5A	Serine/threonine-protein phosphatase 2A 56kDa regulatory subunit alpha isoform	0.63
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	0.51
OTHERS		
KIA1199	KIA1199	1.79
PRSS8	Prostasis precursor	1.75
OBFC1	Oligonucleotide/oligosaccharide-binding fold containing protein 1	0.65
OTUD4	OUT-domain containing protein 4	0.65
FAM133B	FAM133B	0.64
SH3TC2	SH3 domain and tetratricopeptidase repeats-containing protein 2	0.58
C21orf63	Uncharacterized protein C21orf63 precursor	0.57

Data obtained in microarrays can be considered consistent because: (a) the experimental design includes two different cystatin D-expressing clones considered as replicates, as well as mock and wild-type cells as duplicated, a criterion that excluded genes regulated in only one sample; (b) the criteria used to consider genes as regulated were strict enough to eliminate some previously validated genes such as *CTNND1*; and (c) the high percentage of genes validated by quantitative RT-PCR (92%).

Data from microarrays and quantitative RT-PCR show that cystatin D overexpression in HCT116 cells regulates genes involved in diverse cellular functions. We are aware that these are preliminary data and that a second validation using independent sets of RNA is needed. Moreover, validation of these target genes in other cell types as well as functional studies are required to support these results.

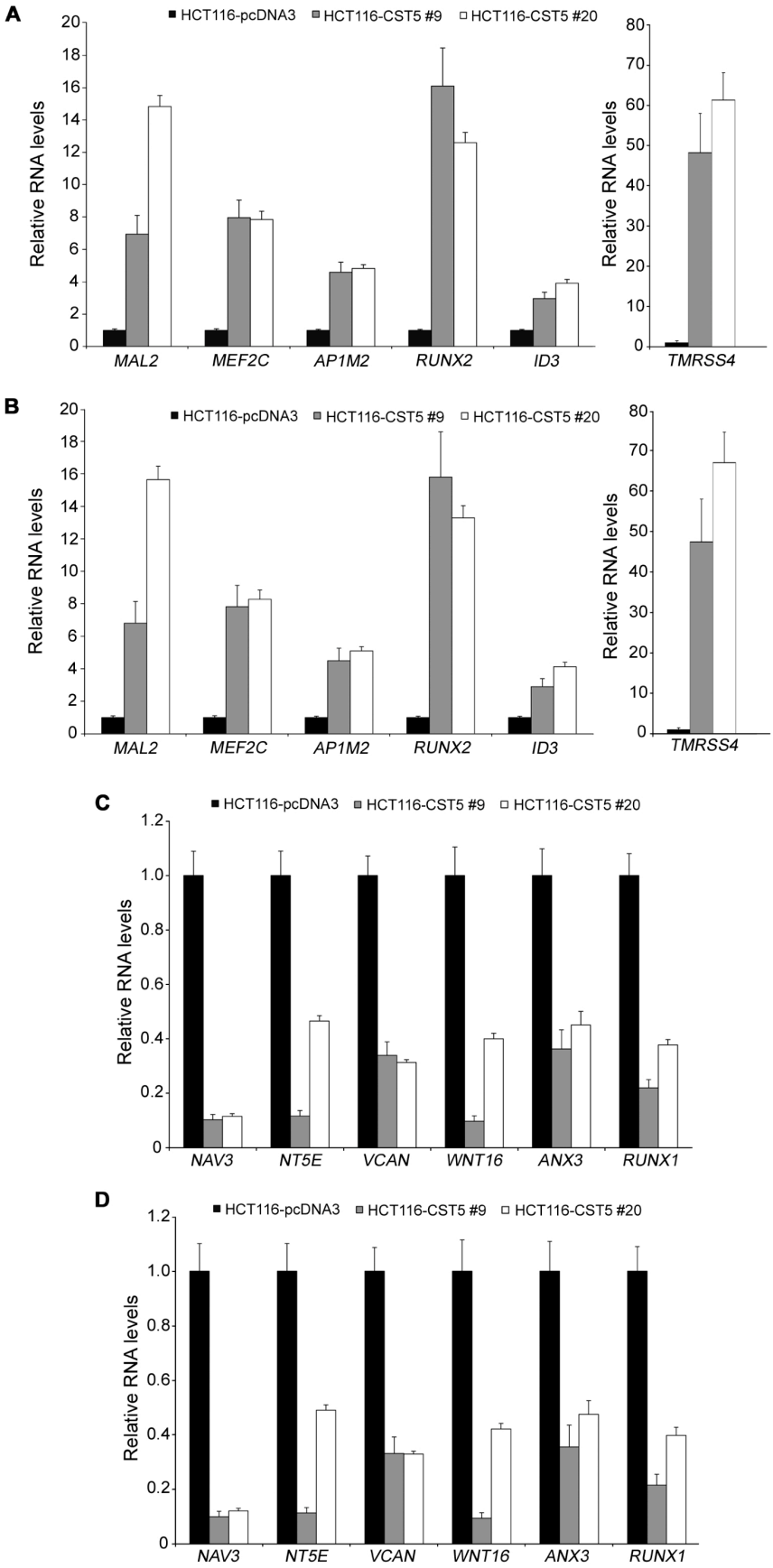

Figure 40. Validation of selected cystatin D-regulated genes in HCT116 cells. Quantitative RT-PCR analysis showing the RNA levels of upregulated **(A** and **B)** and downregulated **(C** and **D)** genes. In each case the genes were normalized both to *18S* **(A** and **C)** and *GAPDH* **(B** and **D)**. Normalized mean values and SD obtained in two independent experiments are shown.

DISCUSSION

CRC is one of the most important neoplasias worldwide in terms of prevalence and mortality. The accepted idea that CRC takes many years to develop enables prevention and early detection. Surgery remains the primary treatment while chemotherapy and/or radiotherapy are recommended depending on the individual patient staging but are rarely curative [76,79,138,191]. 1α,25(OH)$_2$D$_3$ is the most active metabolite of vitamin D$_3$ in human body and has numerous antitumor actions [56]. Previous data from our laboratory and others have shown that 1α,25(OH)$_2$D$_3$ regulates the proliferation and phenotype of colon carcinoma cells through the transcriptional control of a number of target genes and the antagonism of the Wnt/β-catenin signaling pathway [225,227]. These results contribute to explain the higher susceptibility to colon cancer caused by vitamin D deficiency in animal models and the results of epidemiological and clinical studies that sustain a protective and perhaps therapeutic role of 1α,25(OH)$_2$D$_3$ against CRC [85,96,167,205].

Cystatin D is a secreted protease inhibitor found in human saliva and tear fluid [83]. It was first identified as the product of a gene with homology to the cystatin C gene and its sequence contains all regions of relevance for cysteine proteinase inhibitory activity and also the 4 cysteine residues that form disulfide bridges in the other members of Type 2 cystatins [82]. However, the inhibition profile of cystatin D is clearly different from that of other members of its family. Cystatin D is unable to inhibit cathepsin B and pig legumain, displaying a more restricted and specific inhibition profile than its homologues [5]. Probably, due to its unusual expression and inhibition profiles, cystatin D has an unknown biology that has allowed to discover new functions for this protease inhibitor.

Our work has revealed new and unexpected actions of cystatin D that propose it as a candidate tumor suppressor gene in colon cancer. Moreover, in this Thesis we report that 1α,25(OH)$_2$D$_3$ is a strong direct inducer of cystatin D in human colon cancer cells, and that this cathepsin proteases inhibitor partly mediates 1α,25(OH)$_2$D$_3$ antitumor activity.

1. Cystatin D and its new identified tumor suppressor activity

Many proteases, particularly those that degrade ECM, play an important role in tumor development providing an access for tumor cells to the vascular and lymphatic systems, thus favoring angiogenesis and invasion. Moreover, certain proteases also promote tumor cell proliferation and resistance to apoptosis showing that proteolytic enzymes can contribute to all stages of tumor progression [33,63,195]. This is the case of lysosomal cysteine proteases, which have been implicated in multiple steps during tumor progression including early steps of immortalization and transformation, intermediate steps of tumor invasion and angiogenesis, and late steps of metastasis and drug resistance [140]. Thus, cathepsins B and S

seem to contribute to the angiogenic switching and basement membrane degradation in the early preneoplastic lesions. Likewise, cathepsins B, C, L, and Z potentiate the release and activation of pro-growth factors thus favouring tumor growth. On the other hand, cathepsins H and L contribute to invasive growth either through degradation of basement membrane or ECM components or proteolysis of specific target proteins on the cell-surface [91,92,140,161]. All these pro-tumorigenic properties of cathepsins are balanced by the activity of different members of the cystatin family. Thus, cystatin E/M, is a known tumor suppressor gene in human breast cancer that is normally downregulated and epigenetically silenced in this neoplasia [150,270,333]. Moreover, cystatin C has been proposed as a TGFβ receptor antagonist [275,276]. The data reported in this Thesis sustain a similar and, unpredicted role of cystatin D as a candidate tumor suppressor gene.

1.1 Cystatin D inhibits several transformation parameters of human colon cancer cells

Stimulation of cell proliferation, morphological alteration, migration capacity or anchorage independence are transformation parameters that collaborate in tumor progression as well as in metastatic processes. Our results demonstrate that exogenous cystatin D promotes the inhibition of the proliferation, migration and anchorage-independent growth of cultured colon cancer cells and their tumorigenic potential *in vivo*.

We observed that exogenous cystatin D inhibits cell proliferation in all three colon cancer cell lines studied. The finding that addition of recombinant cystatin D protein to cells did not reproduce the effects of endogenous cystatin D indicates that its antitumor effects are mostly exerted intracellularly. Flow cytometry assays showed a slower entry of cystatin D-expressing cells into cell cycle upon synchronization as compared to cystatin D negative cells. This antiproliferative action of cystatin D *in vitro* as well as the antitumor growth *in vivo* on colon cancer cells is most probably linked to the repression of *c-MYC* oncogene. Cystatin D decreased *c-MYC* RNA and protein levels and also the activation of two different *c-MYC* promoter constructs, suggesting a transcriptional regulation. We also observed the inhibition of β-catenin/TCF transcriptional activity. This interference with the Wnt signaling pathway can explain at least partially the downregulation of c-MYC since this oncogene is a known target of Wnt/β-catenin pathway [114]. Remarkably, the suppression of *c-MYC* overexpression is sufficient to cause sustained tumor regression in several model systems, and a threshold level of c-MYC protein is required for tumor maintenance ([268] and references therein). Indeed, c-MYC has a crucial role in cell proliferation. It is known that c-MYC abrogates the transcription of cell cycle checkpoint genes (for example, *GADD45* and *GADD153*), inhibits the function of cyclin-dependent kinase (CDK) inhibitors (reviewed in [46]), and promotes cell

cycle progression by activation of several cyclins (cyclin E1, cyclin A2…), as well as CDK4, E2F1, E2F2 and other proteins [137,172,235]. In addition, and although its regulation by cystatin D seems to be modest, cyclin A2 is one of the genes downregulated by cystatin D overexpression in our global transcriptomic analysis.

Migration and anchorage-independent growth were also inhibited by cystatin D in colon cancer cells. These are characteristics of transformed cells. Moreover, they are involved in the metastatic process allowing the cells to leave the original tumor site and reach other parts of the body. Cathepsins have been related several times with these processes and thus cathepsin B, L, X, C or S participate in tumor formation, growth, migration and invasion [158-160,196,312]. Cystatins have been reported as inhibitors of these processes, and thus, cystatin C and E/M diminish migration and anchorage-independent growth in various cancer cell types [270,275,276,333]. Since cystatin D inhibits some of these proteases, our results are coherent and agree with these findings.

1.2 Role of cystatin D regulating adhesion proteins and their inhibitors

One of the most notable effects of ectopic cystatin D expression is the induction of an adhesive phenotype (HCT116 cells). Molecular analyses showed the induction by cystatin D of adhesion proteins such as E-cadherin, p120-catenin or occludin. These are central proteins for the formation of *adherens* and tight junctions. They are essential for cell-cell interaction in epithelial tissues and their disruption abrogates normal embryonic development and is a common occurrence in metastatic cancers [9,50,59,89,188,210].

Cadherins are of particular importance for the dynamic regulation of adhesive contacts. In adult tissue they are involved in the orderly turnover of rapidly growing tissues such as the lining of the gut and the epidermis [116,293]. Cadherins are also responsible for the maintenance of stable tissue organization to prevent the dissociation and spread of tumor cells [27,39]. Thus, in epithelial derived tumors, loss of cell-cell adhesion is correlated with downregulation of E-cadherin as well as increased proliferation and tumor invasiveness [49,51]. E-cadherin is the key molecule of the cadherin-catenin-cytoskeleton complex, and it is important for establishing and maintaining apico-basal polarity, preserving epithelial cell survival, and controlling proliferation [175]. Perturbation of E-cadherin/catenin complex leads to a dissociation of β-catenin that accumulates in the cytoplasm, translocates to the nucleus and interacts with TCF/LEF transcription factors altering the expression of genes involved in cell proliferation, a signaling pathway activated by Wnt factors [203]. Moreover, disassembly of *adherens junctions* causes loss of E-cadherin mediated adhesion and promotion of cell migration [151,185].

p120-catenin has a crucial role stabilizing the cadherin/catenin complex and it is known that p120-catenin/E-cadherin interaction is required for increased adhesiveness of cells [292].

Due to this role it is not surprising that many cancer types are characterized by loss or dislocalization of p120-catenin (reviewed in [306]). Occludin has also been related with cancer progression and thus, by the regulation of RhoA signaling this adhesion protein participates in cell cycle progression [3,25,327].

The increased expression of these adhesion proteins by cystatin D was associated with the downregulation of EMT genes. EMT is a process whereby epithelial cell layers lose polarity and cell-cell contacts and undergo a dramatic remodeling of the cytoskeleton [291]. A hallmark of EMT is the loss of E-cadherin expression. Loss of E-cadherin is consistently observed at sites of EMT during development and cancer [291]. Several developmentally important genes that induce EMT have been shown to act as E-cadherin repressors. And thus, *SNAIL1, SNAIL2, ZEB1, ZEB2, TWIST* or *E47* bind to the E-boxes at the *CDH1* promoter and repress E-cadherin expression [48,190,208]. These genes also downregulate occludin expression [127]. Therefore, disruption of intercellular junctions causes dissociation of epithelial cells from surrounding cells, acquiring mesenchymal-like characteristics and becoming able to migrate away from the original tissue.

In conclusion, the downregulation of EMT genes linked to enhanced expression of E-cadherin and other adhesive proteins, as well as the inhibition of cell migration and proliferation observed in cystatin D-expressing cells, imply the reversion of the transformed phenotype, hallmark of the EMT process, and the acquisition of epithelial and less aggressive characteristics.

1.3 Are all the antitumor effects of cystatin D mediated by its cathepsin-inhibitory activity?

In this Thesis we show novel and complex actions of cystatin D on colon cancer cells, but the exact molecular mechanism by which cystatin D exerts its actions is unclear. The more reasonable possibility is based on its antiproteolytic activity. However, our results derived from the functional analysis of mutant cystatin D proteins with reduced antiproteolytic activity indicate that cystatin D exerts its antimigratory effects as well as the regulation of adhesion proteins and their inhibitors through cathepsin inhibition, while its antiproliferative effect on colon cancer cells seems to be independent of this activity (Figure 41).

It is known that one of the mechanisms to downregulate E-cadherin expression is based on the proteolytic cleavage of its ectodomain by proteases such as MMP-3 and -7 or

ADAM10, among others [185,211]. In line with this, the cystatin D targets cathepsin L and S have been reported to cleave E-cadherin *in vitro* and possibly in Rip1-Tag2 (RT2) mice* [92].

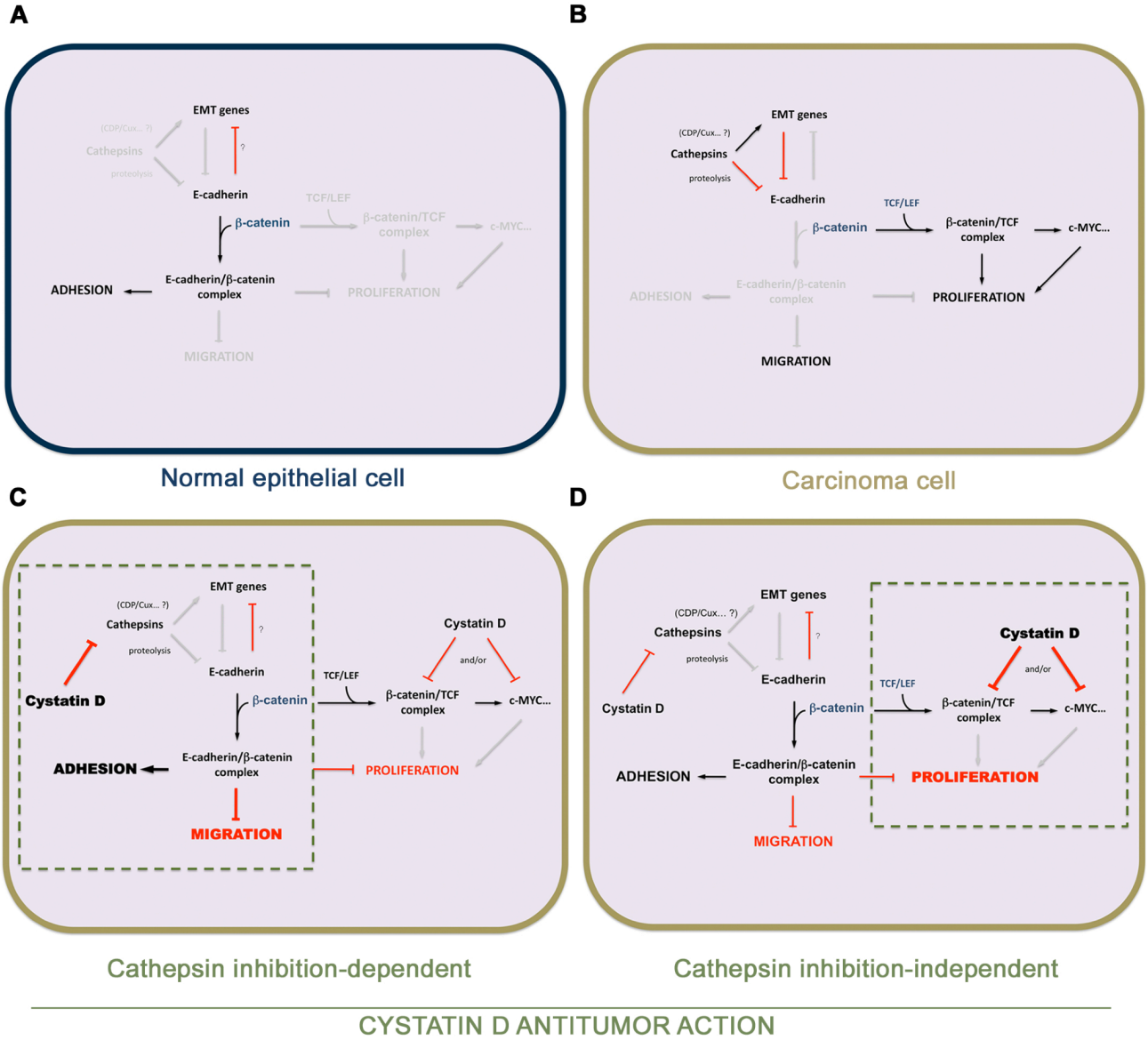

Figure 41. Schemes showing the interplay between E-cadherin, EMT genes, cathepsins and β-catenin in the control of cell adhesion, proliferation and migration. **(A)** Normal epithelial cell. **(B)** Carcinoma cell. **(C)** Blockade of colon carcinoma cell migration by cystatin D *via* cathepsin inhibition-dependent mechanisms. **(D)** Blockade of colon carcinoma cell proliferation by cathepsin inhibition-independent mechanisms. Grayed out represent the processes inactive in each situation. Processes inhibited are highlighted in red and those induced are highlighted in black.

* RIP-Tag mice carry the SV40 early region encoding the large T (Tag) and small t oncoproteins under the control of the rat insulin gene regulatory region (RIP). Tag abrogates the functions of the retinoblastoma and p53 tumor suppressors in the pancreatic islet β cells, leading to the formation of β cell tumors (insulinomas) in every mouse inheriting the hybrid oncogene.

RT2 transgenic mice, that are models of multistage cancer, develop multiple pancreatic islet tumors, and E-cadherin downregulation has been functionally implicated in acquisition of the invasive growth phenotype in this pathway [238]. Thus, cathepsin L and S knockout (*CtsL*$^{-/-}$ RT2, and *CtsS*$^{-/-}$ RT2) mice maintained E-cadherin protein levels in comparison with control RT2 mice and this is associated with a pronounced reduction in tumor invasion *in vivo* [92] In addition, a shorter isoform of cathepsin L lacking the signal peptide is present within the nucleus of mouse 3T3 and human breast cancer cells and modulates the proteolytic processing of the CCAAT-displacement protein/cut homeobox (CDP/Cux) transcription factor generating a p110 isoform [97]. The p110 CDP/Cux isoform has been related with the regulation of cell cycle progression [264]. Moreover, it has recently been showed that p110 CDP/Cux binds to and activates the *SNAI1* and *SNAI2* gene promoters, cooperating with these transcription factors in the repression of E-cadherin, thereby causing disorganization of cell-cell junctions [146].

In human colon cancer cells, however, we did not detect the E-cadherin cleaved fragments (64 and 30-35 kDa) that are generated by cathepsin L. Likewise, we found no changes in the expression of CDP/Cux polypeptides associated with cystatin D expression (data not shown). These differences may be due to species- and/or cell type- specific activity of cathepsin L. Due to the opposite regulation of *CDH1* RNA levels observed between wild-type and mutant cystatin D protein-expressing cells, as well as in that of EMT genes, it is reasonable that another still uncharacterized cathepsin-mediated mechanism is taking place in our system. Alternatively, the recent description by Weinberg's group [184,219] that E-cadherin loss in breast cancer cells promotes cancer stem cell–like properties and metastasis through the induction of EMT suggests that cystatin D may primarily affect *CDH1* RNA transcription or stability.

In addition, cystatin D has effects unrelated to the inhibition of cathepsins such us proliferation arrest or the downregulation of c-MYC expression. Cystatin D may exert these actions by interacting with other non-characterized proteins. In recent years, cystatins have been proposed to play important roles in tumor progression apparently unrelated to their cathepsin-inhibitory action, and numerous studies involving cystatins C and E/M have described this dual function [270,276]. Thus, cystatin E/M, similarly to cystatin D, exerts its antiproliferative effect in breast cancer cells independently of cathepsin inhibition [270]. Likewise, cystatin C, *via* a cathepsin-independent mechanism, antagonizes TGFβ signaling pathway by interacting physically with the TGFβ type II receptor and antagonizing the binding of TGFβ [276]. Moreover, another illustrative example in this regard is that of the tissue inhibitor of metalloproteinases (TIMP)-2, which inhibits mitogenesis and angiogenesis at least in part by metalloproteinase-independent mechanisms [282].

The finding that mutant cystatin D proteins with reduced antiproteolytic activity inhibit cell proliferation but not cell migration makes plausible that both, cathepsin inhibition-dependent

and -independent mechanisms are responsible for cystatin D antitumor activity. This novel dual action suggests future studies aimed to identify new mechanisms or cystatin D-interacting proteins.

2. Cystatin D is an important mediator of 1α,25(OH)$_2$D$_3$ action in colon cancer

Numerous studies have shown that 1α,25(OH)$_2$D$_3$ is a major transcriptional regulator of gene expression. Identification of novel 1α,25(OH)$_2$D$_3$ target genes is necessary to understand the anti-cancer role of this hormone. An important finding of this Thesis has been to identify and characterize the direct regulation of cystatin D expression by 1α,25(OH)$_2$D$_3$ and describe the role of this inhibitor as mediator of 1α,25(OH)$_2$D$_3$ actions against colon cancer.

2.1 1α,25(OH)$_2$D$_3$ induces cystatin D expression in colon cancer cell lines

Our results demonstrate that 1α,25(OH)$_2$D$_3$ increases *CST5* RNA and protein expression in human colon cancer cells. They validate previous results of our laboratory using oligonucleotide microarrays that showed an increase in the level of *CST5* RNA following 1α,25(OH)$_2$D$_3$ treatment of SW480-ADH cells [227]. Previously, cystatin E/M was identified as a target of the 1α,25(OH)$_2$D$_3$ analog EB1089 in squamous carcinoma cells. The upregulation of cystatin E/M by the hormone was confirmed by Northern blot and immunofluorescence analyses, but no functional studies has been reported yet [176]. In addition, cystatin A is induced by 1α,25(OH)$_2$D$_3$ in normal keratinocytes. This regulation is mediated by inhibition of Raf-1/MEK1/ERK signaling pathway *via* a non-genomic response [287]. Moreover, 1α,25(OH)$_2$D$_3$ also regulates the expression of other protease inhibitors such as TIMPs or plasminogen activators inhibitors (PAIs). Thus, TIMP-1 is upregulated by 1α,25(OH)$_2$D$_3$ in breast and prostate cancer cells as well as PAI-1 in breast cancer cells [14,154].

The finding that the increase of cystatin D by 1α,25(OH)$_2$D$_3$ was abrogated by the transcription inhibitor actinomycin D but not by the translation inhibitor cycloheximide indicated a direct transcriptional effect of 1α,25(OH)$_2$D$_3$. Moreover, transactivation and ChIP assays showed that this activation is mediated by the binding of VDR to a cluster of sites located close to the transcription start site at the cystatin D promoter. These studies also showed that a functional VDR was needed for this activation. Recently, it has been proposed that isolated, simple VDREs may be not functional *in vivo*, and that, contrarily, transcription factor binding site clusters or modules contribute to chromatin decondensation allowing a major accessibility of transcription factors to a promoter [41]. Our results fit with this theory and

can explain why the region of *CST5* promoter with a greater number of VDREs is most activated by 1α,25(OH)$_2$D$_3$ with lower or no contribution of isolated VDREs.

We have also shown that 1α,25(OH)$_2$D$_3$ regulates cystatin D expression in other colon cancer cell lines, which supports a general regulation of this inhibitor by 1α,25(OH)$_2$D$_3$ in colon cancer. In addition, the finding that cystatin D was also regulated by EB1089 in xenografted mice sustains a role for 1α,25(OH)$_2$D$_3$ in the regulation of cystatin D *in vivo*.

2.2 Cystatin D mimics the effect of 1α,25(OH)$_2$D$_3$ treatment

The number and importance of the actions of cystatin D and its strong, rapid and direct transcriptional regulation by 1α,25(OH)$_2$D$_3$ indicate that cystatin D is an important mediator of 1α,25(OH)$_2$D$_3$ action in colon cancer cells at least *in vitro*. This is further emphasized by the results obtained after expressing an exogenous cystatin D or by down-regulating the endogenous *CST5* gene by means of shRNA in SW480-ADH cells. In these cells ectopic cystatin D expression inhibited cell proliferation at similar levels than that observed only with the hormone and, in addition, 1α,25(OH)$_2$D$_3$ treatment enhanced this inhibition. It is known that one antitumor actions of 1α,25(OH)$_2$D$_3$ is the inhibition of cell proliferation, and this effect is mediated in part by the inhibition of c-MYC expression [34,228,229]. c-MYC protein levels decreased upon expression of exogenous cystatin D similarly to what happens in cells treated with 1α,25(OH)$_2$D$_3$. Additionally, 1α,25(OH)$_2$D$_3$ enhances the inhibition of c-MYC caused by cystatin D. These results correlate with the effects observed on cell proliferation, explaining the additive effect of 1α,25(OH)$_2$D$_3$ and cystatin D in these cells. 1α,25(OH)$_2$D$_3$ also regulates other genes involved in cell cycle progression such as *p21$^{WAF1/CIP1}$*, *p27^{KIP1}*, *c-JUN* and others (reviewed in [95]), which can explain why the treatment of cystatin D-expressing cells with 1α,25(OH)$_2$D$_3$ lead to a greater inhibition of cell proliferation. The finding that cystatin D knockdown completely abrogates the 1α,25(OH)$_2$D$_3$-mediated inhibition of cell proliferation suggests that cystatin D is required to the antiproliferative effect of 1α,25(OH)$_2$D$_3$.

All effects analyzed in cystatin D-expressing SW480-ADH cells after treatment with 1α,25(OH)$_2$D$_3$ were inversely regulated by *CST5* shRNA. In this way, cystatin D overexpression increased the induction of E-cadherin by 1α,25(OH)$_2$D$_3$ while the opposite effect was observed in *CST5* shRNA cells. Moreover, *CST5* knockdown abrogates in part the phenotypic change induced by 1α,25(OH)$_2$D$_3$, an effect that may be explained by the reduction of E-cadherin-mediated intercellular adhesion.

Altogether these data suggest a critical contribution of cystatin D mediating the antitumor actions of 1α,25(OH)$_2$D$_3$. 1α,25(OH)$_2$D$_3$ promotes apoptosis and induces cell differentiation,

and inhibits migration and invasion as a result of the regulation of a high number of target genes,. Among them, *cystatin D* contributes by cathepsin-dependent and independent mechanisms to several of these anticancer activities (Figure 42).

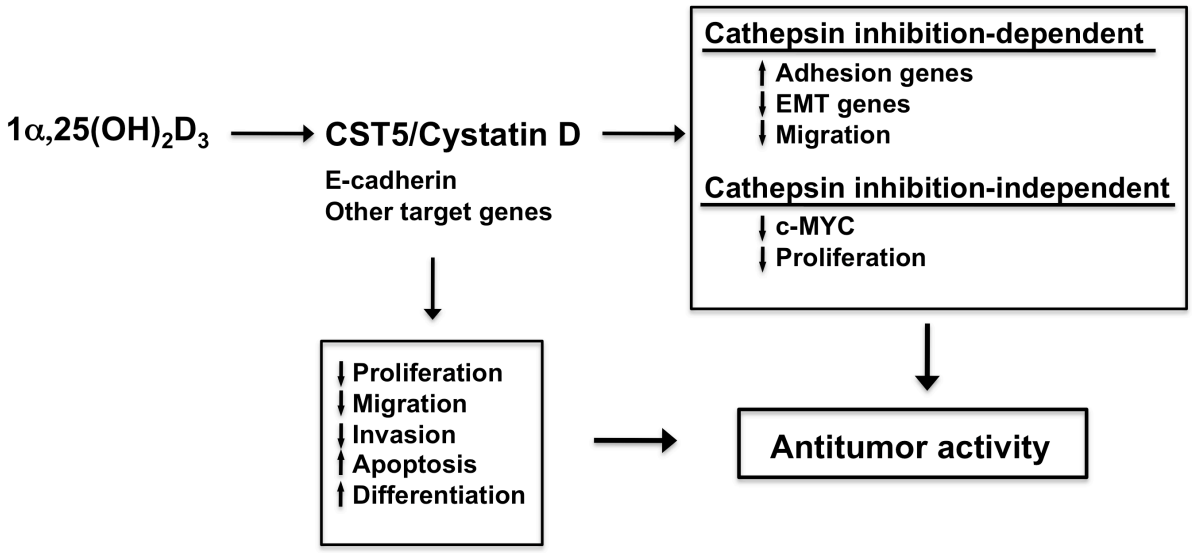

Figure 42. Scheme showing the role of cystatin D as mediator of 1α,25(OH)$_2$D$_3$ antitumor activity. Among the genes regulated by 1α,25(OH)$_2$D$_3$ cystatin D play important roles mediating the hormone antitumor actions through cathepsin-dependent and cathepsin-independent mechanisms.

3. Cystatin D expression in human colon cancer

Results obtained in this Thesis using human patients' samples strongly indicate that *CST5* gene is downregulated during colon tumorigenesis associated with tumor dedifferentiation. Previously, cystatin D expression had only been detected in saliva and tears [83]. The loss of cystatin D expression during colon tumorigenesis associated to dedifferentiation supports a role for this protein in the control of cell phenotype *in vivo*, and emphasizes its nature as a candidate tumor suppressor. Analogously, cystatin E/M has recently been shown to be downregulated in glioma, breast, prostate and cervical cancers [150,250,251,265,310]. Likewise, cystatin C is downregulated in approximately 50% of human malignancies, particularly in cancers of the stomach, uterus, colon, and kidney ([276] and references therein). Moreover, cystatin A is lost along the transformation of myoepithelial cells during tumorigenesis in most breast and prostate cancer, as well as in head and neck squamous cell carcinomas (HNSCC) and in brain tumors [66,162,165,174,272,283,284]. Furthermore, high level of cystatin B correlates with increased probability of survival in patients with non-small cell lung cancer

and is downregulated in many breast cancers [165,166,173,316]. It is known that cystatins are epigenetically silenced through DNA methylation-dependent mechanisms in several forms of cancer, including breast, pancreatic, brain, and lung (reviewed in [259]). For this reason we studied whether the same mechanism occurred in case of cystatin D. We used Methyl Primer Express v1.0 software (Applied Biosystems) to identify CpG islands in an interval of 2.0 kb upstream and 1.5 kb downstream from the first ATG codon. The search was negative. Therefore, *CST5* gene most probably lacks CpG islands in the vicinity of its promoter and thus the silencing mechanism based on CpG islands hypermethylation is not responsible for cystatin D repression.

We also found a correlation between cystatin D expression and VDR protein levels in colon biopsies supporting a role of $1\alpha,25(OH)_2D_3$ in the regulation of cystatin D in the organism. Remarkably, VDR expression is associated with cell differentiation, absence of node involvement and favourable prognosis in colorectal cancer [52,71,236,309], which is in line with the loss of cystatin D in poorly differentiated tumors. Also the direct correlation between the expression of cystatin D and E-cadherin, which is a marker and crucial regulator of the epithelial phenotype and invasion, further supports the relation of cystatin D with tumor differentiation.

Together, our findings reveal an unpredicted activity of cystatin D as tumor suppressor. According to the results with tumor biopsies, cystatin D also plays putative protective effects in humans. Furthermore, our results illustrate a novel mechanism of the anticancer action of the most active vitamin D metabolite and rationalize its preventive and therapeutic use against colon cancer.

4. Gene expression profile induced by cystatin D

Our observations that cystatin D is able to reduce cell proliferation and migration, as well as to increase cell adhesion suggest that it might behave like an autocrine factor regulating cellular properties. Some of these effects of cystatin D are independent on the inhibition of cathepsins activity. To explore this hypothesis, we have used DNA microarray analysis to define the gene expression profile induced by cystatin D. The categories of cell adhesion, transcription, and signal transduction were the most represented among genes whose RNA levels change by exogenous cystatin D expression: 35 out of 69 regulated genes were related with these processes.

To our knowledge, this is the second study showing that a cystatin has the capacity to modify gene expression. Only one previous study had shown that cystatin E/M alters the gene expression profile of the MDA-MB-435S cancer cell line [278]. The comparison with this

work reveals some similarities between the two cystatins. Thus, both regulate a similar number of genes (61 in case of cystatin E/M), with more genes downregulated than upregulated (41 *versus* 20 for cystatin E/M and 46 *versus* 23 for cystatin D). Moreover, in both cases a great number of target genes are related with signal transduction and/or transcription [278]. Although these studies are performed in different systems (breast and colon cancer) and imply different molecules, the cellular functions regulated by both inhibitors are similar. Hence, it is possible that cystatins may be implicated in the control of overlapping processes.

Of note, cystatin D expression exerts no compensatory effects on the expression of lysosomal cysteine proteases or another members of the cystatin superfamily.

Among the genes regulated by cystatin D there are several transcription factors. Two members of the Runt-related transcription factors (RUNXs) are regulated in an opposite fashion. Thus, *RUNX2* is upregulated by cystatin D whereas *RUNX1* is downregulated. *RUNX2* is known for its role in the differentiation of osteoblasts and chondrocytes from mesenchymal precursors [67,156,223,326]. Osteocalcin (*OCN*) is the most established target gene for *RUNX2* [13,224]. It is also known the key role that *RUNX2* plays in the $1\alpha,25(OH)_2D_3$-mediated upregulation of *OCN* in osteoblasts, by stabilizing through direct protein-protein interaction the binding of VDR to the VDREs present in the *OCN* promoter [231,232]. Recently, *RUNX2* has been proposed as a tumor suppressor [29,328] and some of the target genes that mediate its putative tumor suppressor activity are the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ or the pro-apoptotic protein BAX, both known $1\alpha,25(OH)_2D_3$ targets [65,328]. *RUNX1* is essential for the generation of hematopoietic stem cells and is involved in human leukemia [37,54]. It is also known that *RUNX1* induces transformation of 3T3 fibroblasts and stimulation of cell cycle progression [26,40,163,181,285], and recently, it has been related with lymphoma development ([30] and references therein). These two genes, *RUNX1* and *RUNX2*, have been validated by quantitative RT-PCR as cystatin D targets and further studies are necessary to elucidate the role that play in cystatin D actions.

Cystatin D also regulates several genes related with cell adhesion such as *CDH1*, *CLDN7* (upregulated), *VCAN* or *VIM* (downregulated). *CDH1* has been already validated along this Thesis as cystatin D target by quantitative RT-PCR and western blot, and the probable role of E-cadherin in cystatin D actions has been already discussed. *CLDN7*/claudin-7 encodes a member of the tight junctions that has an important role in the maintenance of the epithelial differentiated phenotype [157]. It has been showed that ductal breast carcinoma and HNSCC have reduced expression of *CLDN7* [4,155]. Moreover, reduced expression of *CLDN7* is correlated with invasion and metastasis of squamous cell carcinoma of the esophagus [302]. Likewise, *CLDN7* expression diminishes during colon cancer progression and is correlated with liver metastasis [201,222]. *VCAN*/versican, encodes a chondroitin sulfate proteoglycan that

is one of the main components of the extracellular matrix. Recent data suggest that *VCAN* modulates cell adhesion, proliferation, and migration. Hence, it plays a central role in tissue development and maintenance as well as in a number of pathologic processes (reviewed in [257]). Thus, increased expression of *VCAN* is strongly associated with poor outcome in many types of cancer [257]. *VIM*/vimentin encodes an intermediate filament protein, well-known marker for EMT that is expressed in mesenchymal cells [88]. Several studies have demonstrated a functional contribution of vimentin to epithelial cell invasion and migration in numerous cell types [60,61,87,115,336]. Moreover, a correlation has been suggested between vimentin expression and the perturbation of E-cadherin-mediated cell adhesion during cell migration. For instance, several studies comparing highly invasive breast or lung cell lines revealed that the loss of E-cadherin is associated with vimentin expression [243,277]. Due to these data the three genes *CLDN7*, *VCAN*, and *VIM* are attractive targets of cystatin D. *VCAN* has been already validated by quantitative RT-PCR while future validation is needed for *CLDN7* and *VIM*.

Among the genes regulated by cystatin D that are related with signal transduction it is worth mentioning *NAV3*. This gene is strongly downregulated, as validated by quantitative RT-PCR. Little is known about *NAV3*, a member of the neuron navigator family of homologues of *unc-53*, a gene involved in axon guidance in *Caenorhabditis elegans* [182]. However, it was recently included by Vogelstein's group in the genomic landscape of human breast and colorectal cancers [318]. In these studies, the authors propose that the genomic landscape of these two neoplasias are composed of a handful of commonly mutated gene "mountains" and a much larger number of gene "hills" that are mutated at low frequency. *NAV3* is proposed as one of the "hill" genes implied in colorectal cancer [318], but no functional studies have showed relation of *NAV3* with colon cancer yet. However, the strong regulation that cystatin D seems to exert over this gene together with its plausible role in colon neoplasia makes the study highly attractive.

Notably, all the candidate cystatin D target genes related with metabolism are downregulated. *NT5E* and *TXN* are two of these genes and both are overexpressed in a variety of tumors. *NT5E*/CD73 encodes an enzyme that catalyzes the dephosphorylation of ribo- and deoxyribo-nucleotide 5'-monophosphates to their corresponding nucleosides, and is highly expressed in carcinomas of colon, lung, pancreas and ovary. Moreover, its expression level has been associated with tumor neovascularization, invasiveness, and metastasis, and with shorter patient survival [23,313]. *TXN*/thioredoxin encodes a protein related with the regulation of cellular redox homeostasis. *TXN* contributes to many of the hallmarks of cancer including increased proliferation, resistance to cell death and increased angiogenesis, and is overexpressed in lung, breast, pancreatic, cervical, gastric and colon carcinomas [248,249].

Remarkably, a 4% of genes regulated by cystatin D are involved in RNA splicing. *RBM35A* is one of these genes, and a recently published study proposes it as a novel tumor suppressor for colorectal cancer [171]. The authors propose that *RBM35A* suppresses the malignant potential on LS180 colon cancer cells through a mechanism of translational regulation of gene expression. The finding that cystatin D induces its expression on colon cancer cells suggests a possible role of this gene in the antitumor activity of cystatin D.

Further studies are required to corroborate if these candidate target genes play a role in cystatin D actions and, if so, to elucidate their mechanism and importance.

CONCLUSIONS

1. $1\alpha,25(OH)_2D_3$ induces the expression of *CST5* gene by direct activation of its promoter causing an increase in cystatin D RNA and protein levels in human colon cancer cells.

2. Ectopic cystatin D expression mimics partially the effects of $1\alpha,25(OH)_2D_3$ in SW480-ADH cells. *CST5* knock-down by stable shRNA expression attenuates these effects.

3. Exogenous cystatin D inhibits several transformation processes as proliferation, migration, and anchorage-independent growth of cultured colon cancer cells and their tumorigenesis in immunodeficient mice.

4. Ectopic cystatin D expression represses *c-MYC* oncogene and inhibits Wnt/β-catenin signaling, which play a central role during CRC progression.

5. Exogenous cystatin D induces an adhesive phenotype by increasing E-cadherin and other intercellular adhesion proteins expression and inhibits several inducers of epithelial-mesenchymal transition such as *SNAI1, SNAI2, ZEB1* and *ZEB2*.

6. Mutant cystatin D proteins with reduced antiproteolytic activity maintain the antiproliferative but not the migration-inhibitory activity.

7. Cystatin D expression decreases during human colorectal tumorigenesis associated with tumor dedifferentiation and correlates with VDR and E-cadherin loss.

8. Exogenous cystatin D distinctly alters the expression of a number of genes encoding proteins with different cellular functions.

In summary, cystatin D is a novel $1\alpha,25(OH)_2D_3$ target in human colon cancer cells that behaves as a candidate tumor suppressor contributing to its protective effects against this neoplasia by antiproteolytic-dependent and -independent mechanisms.

CONCLUSIONES

1. La 1α,25$(OH)_2D_3$ induce, en células humanas de cáncer de colon, la expresión del gen *CST5* mediante la activación directa de su promotor, aumentando sus niveles de RNA y proteína.

2. La expresión ectópica de cistatina D mimetiza parcialmente los efectos de la 1α,25$(OH)_2D_3$ en las células SW480-ADH, mientras que el silenciamiento del gen *CST5* mediante la expresión estable de shRNA atenúa estos efectos.

3. La cistatina D exógena inhibe diversos procesos de transformación tumoral como proliferación, migración y crecimiento independiente de anclaje a sustrato de células de cáncer colon, así como su capacidad tumorogénica en ratones inmunosuprimidos.

4. La expresión ectópica de cistatina D reprime al oncogén *c-MYC* e inhibe la ruta de señalización Wnt/β-catenina, los cuales juegan un papel crucial durante la progresión del CRC.

5. La cistatina D exógena induce un fenotipo adhesivo aumentando la expresión de E-cadherina y otras proteínas de adhesión intercelular e inhibe la expresión de varios genes inductores de transición epitelio-mesénquima como *SNAI1, SNAI2, ZEB1* y *ZEB2*.

6. Mutantes de cistatina D con reducida actividad antiproteolítica mantienen la actividad antiproliferativa pero no la inhibición de la migración celular.

7. La expresión de cistatina D disminuye durante la tumorogénesis colorrectal humana asociada con la desdiferenciación tumoral y se correlaciona con la pérdida de expresión de VDR y E-cadherina.

8. La cistatina D exógena altera la expresión de un cierto número de genes que codifican proteínas implicadas en diferentes funciones celulares.

En resumen, la cistatina D es una nueva diana de la 1α,25$(OH)_2D_3$ en células humanas de cáncer de colon, que se comporta como un posible supresor tumoral contribuyendo a los efectos de la 1α,25$(OH)_2D_3$ frente a esta neoplasia mediante mecanismos dependientes e independientes de su actividad antiproteolítica.

REFERENCES

1. Abrahamson M: Cystatins. **Methods Enzymol 244:**685-700, 1994
2. Abrahamson M, Barrett AJ, Salvesen G, Grubb A: Isolation of six cysteine proteinase inhibitors from human urine. Their physicochemical and enzyme kinetic properties and concentrations in biological fluids. **J Biol Chem 261:**11282-11289, 1986
3. Aijaz S, D'Atri F, Citi S, Balda MS, Matter K: Binding of GEF-H1 to the tight junction-associated adaptor cingulin results in inhibition of Rho signaling and G1/S phase transition. **Dev Cell 8:**777-786, 2005
4. Al Moustafa AE, Alaoui-Jamali MA, Batist G, Hernandez-Perez M, Serruya C, Alpert L, et al: Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous carcinoma cells. **Oncogene 21:**2634-2640, 2002
5. Alvarez-Fernandez M, Liang YH, Abrahamson M, Su XD: Crystal structure of human cystatin D, a cysteine peptidase inhibitor with restricted inhibition profile. **J Biol Chem 280:**18221-18228, 2005
6. Anastasiadis PZ, Moon SY, Thoreson MA, Mariner DJ, Crawford HC, Zheng Y, et al: Inhibition of RhoA by p120 catenin. **Nat Cell Biol 2:**637-644, 2000
7. Andoh A, Bamba S, Brittan M, Fujiyama Y, Wright NA: Role of intestinal subepithelial myofibroblasts in inflammation and regenerative response in the gut. **Pharmacol Ther 114:**94-106, 2007
8. Arends JW: Molecular interactions in the Vogelstein model of colorectal carcinoma. **J Pathol 190:**412-416, 2000
9. Assemat E, Bazellieres E, Pallesi-Pocachard E, Le Bivic A, Massey-Harroche D: Polarity complex proteins. **Biochim Biophys Acta 1778:**614-630, 2008
10. Baeke F, van Etten E, Gysemans C, Overbergh L, Mathieu C: Vitamin D signaling in immune-mediated disorders: Evolving insights and therapeutic opportunities. **Mol Aspects Med 29:**376-387, 2008
11. Balbin M, Freije JP, Abrahamson M, Velasco G, Grubb A, Lopez-Otin C: A sequence variation in the human cystatin D gene resulting in an amino acid (Cys/Arg) polymorphism at the protein level. **Hum Genet 90:**668-669, 1993
12. Balbin M, Hall A, Grubb A, Mason RW, Lopez-Otin C, Abrahamson M: Structural and functional characterization of two allelic variants of human cystatin D sharing a characteristic inhibition spectrum against mammalian cysteine proteinases. **J Biol Chem 269:**23156-23162, 1994
13. Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS, et al: Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. **J Cell Biochem 66:**1-8, 1997
14. Bao BY, Yeh SD, Lee YF: 1alpha,25-dihydroxyvitamin D3 inhibits prostate cancer cell invasion via modulation of selective proteases. **Carcinogenesis 27:**32-42, 2006
15. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al: Identification of stem cells in small intestine and colon by marker gene Lgr5. **Nature 449:**1003-1007, 2007
16. Barrallo-Gimeno A, Nieto MA: The Snail genes as inducers of cell movement and survival: implications in development and cancer. **Development 132:**3151-3161, 2005
17. Barrett AJ: The cystatins: a diverse superfamily of cysteine peptidase inhibitors. **Biomed Biochim Acta 45:**1363-1374, 1986
18. Barrett AJ, Fritz H, Grubb A, Isemura S, Jarvinen M, Katunuma N, et al: Nomenclature and classification of the proteins homologous with the cysteine-proteinase inhibitor chicken cystatin. **Biochem J 236:**312, 1986
19. Basuroy S, Seth A, Elias B, Naren AP, Rao R: MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide. **Biochem J 393:**69-77, 2006
20. Basuroy S, Sheth P, Kuppuswamy D, Balasubramanian S, Ray RM, Rao RK: Expression of kinase-inactive c-Src delays oxidative stress-induced disassembly and accelerates calcium-mediated reassembly of tight junctions in the Caco-2 cell monolayer. **J Biol Chem 278:**11916-11924, 2003
21. Batlle E, Bacani J, Begthel H, Jonkheer S, Gregorieff A, van de Born M, et al: EphB receptor activity suppresses colorectal cancer progression. **Nature 435:**1126-1130, 2005
22. Batlle E, Sancho E, Francí C, Domínguez D, Monfar M, Baulida J, et al: The transcription factor Snail is a repressor of *E-cadherin* gene expression in epithelial tumour cells. **Nat Cell Biol 2:**84-89, 2000

23. Bavaresco L, Bernardi A, Braganhol E, Cappellari AR, Rockenbach L, Farias PF, et al: The role of ecto-5'-nucleotidase/CD73 in glioma cell line proliferation. **Mol Cell Biochem 319:**61-68, 2008
24. Belleli A, Shany S, Levy J, Guberman R, Lamprecht SA: A protective role of 1,25-dihydroxyvitamin D_3 in chemically induced rat colon carcinogenesis. **Carcinogenesis 13:**2293-2298, 1992
25. Benais-Pont G, Punn A, Flores-Maldonado C, Eckert J, Raposo G, Fleming TP, et al: Identification of a tight junction-associated guanine nucleotide exchange factor that activates Rho and regulates paracellular permeability. **J Cell Biol 160:**729-740, 2003
26. Bernardin F, Friedman AD: AML1 stimulates G1 to S progression via its transactivation domain. **Oncogene 21:**3247-3252, 2002
27. Berx G, Nollet F, van Roy F: Dysregulation of the E-cadherin/catenin complex by irreversible mutations in human carcinomas. **Cell Adhes Commun 6:**171-184, 1998
28. Bettoun DJ, Buck DW, Lu J, Khalifa B, Chin WW, Nagpal S: A vitamin D receptor-Ser/Thr phosphatase-p70 S6 kinase complex and modulation of its enzymatic activities by the ligand. **J Biol Chem 277:**24847-24850, 2002
29. Blyth K, Cameron ER, Neil JC: The RUNX genes: gain or loss of function in cancer. **Nat Rev Cancer 5:**376-387, 2005
30. Blyth K, Slater N, Hanlon L, Bell M, Mackay N, Stewart M, et al: Runx1 promotes B-cell survival and lymphoma development. **Blood Cells Mol Dis 43:**12-19, 2009
31. Bolstad BM, Irizarry RA, Astrand M, Speed TP: A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. **Bioinformatics 19:**185-193, 2003
32. Booth C, Potten CS: Gut instincts: thoughts on intestinal epithelial stem cells. **J Clin Invest 105:**1493-1499, 2000
33. Borgono CA, Diamandis EP: The emerging roles of human tissue kallikreins in cancer. **Nat Rev Cancer 4:**876-890, 2004
34. Brelvi ZS, Studzinski GP: Inhibition of DNA synthesis by an inducer of differentiation of leukemic cells, 1 alpha, 25 dihydroxy vitamin D3, precedes down regulation of the c-myc gene. **J Cell Physiol 128:**171-179, 1986
35. Buhling F, Peitz U, Kruger S, Kuster D, Vieth M, Gebert I, et al: Cathepsins K, L, B, X and W are differentially expressed in normal and chronically inflamed gastric mucosa. **Biol Chem 385:**439-445, 2004
36. Buitrago C, González Pardo V, de Boland AR: Nongenomic action of $1\alpha,25(OH)_2$-vitamin D_3. Activation of muscle cell PLCγ through the tyrosine kinase c-Src and PtdIns 3-kinase. **Eur J Biochem 269:**2506-2515, 2002
37. Cameron ER, Neil JC: The Runx genes: lineage-specific oncogenes and tumor suppressors. **Oncogene 23:**4308-4314, 2004
38. Campbell MJ, Adorini L: The vitamin D receptor as a therapeutic target. **Expert Opin Ther Targets 10:**735-748, 2006
39. Cano A, Pérez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, et al: The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. **Nat Cell Biol 2:**76-83, 2000
40. Cao W, Britos-Bray M, Claxton DF, Kelley CA, Speck NA, Liu PP, et al: CBF beta-SMMHC, expressed in M4Eo AML, reduced CBF DNA-binding and inhibited the G1 to S cell cycle transition at the restriction point in myeloid and lymphoid cells. **Oncogene 15:**1315-1327, 1997
41. Carlberg C, Seuter S: A genomic perspective on vitamin D signaling. **Anticancer Res 29:**3485-3493, 2009
42. Chambon P: A decade of molecular biology of retinoic acid receptors. **FASEB J 10:**940-954, 1996
43. Charafe-Jauffret E, Monville F, Bertucci F, Esterni B, Ginestier C, Finetti P, et al: Moesin expression is a marker of basal breast carcinomas. **Int J Cancer 121:**1779-1785, 2007
44. Christakos S, Dhawan P, Liu Y, Peng X, Porta A: New insights into the mechanisms of vitamin D action. **J Cell Biochem 88:**695-705, 2003
45. Chung I, Han G, Seshadri M, Gillard BM, Yu WD, Foster BA, et al: Role of vitamin D receptor in the antiproliferative effects of calcitriol in tumor-derived endothelial cells and tumor angiogenesis in vivo. **Cancer Res 69:**967-975, 2009
46. Claassen GF, Hann SR: Myc-mediated transformation: the repression connection. **Oncogene 18:**2925-2933, 1999

47. Clevers H: Wnt/beta-catenin signaling in development and disease. **Cell 127:**469-480, 2006
48. Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, et al: The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. **Mol Cell 7:**1267-1278, 2001
49. Conacci-Sorrell M, Zhurinsky J, Ben-Ze'ev A: The cadherin-catenin adhesion system in signaling and cancer. **J Clin Invest 109:**987-991, 2002
50. Costa M, Raich W, Agbunag C, Leung B, Hardin J, Priess JR: A putative catenin-cadherin system mediates morphogenesis of the Caenorhabditis elegans embryo. **J Cell Biol 141:**297-308, 1998
51. Cowin P, Rowlands TM, Hatsell SJ: Cadherins and catenins in breast cancer. **Curr Opin Cell Biol 17:**499-508, 2005
52. Cross HS, Bajna E, Bises G, Genser D, Kállay E, Pötzi R, et al: Vitamin D receptor and cytokeratin expression may be progression indicators in human colon cancer. **Anticancer Res 16:**2333-2337, 1996
53. Davis MA, Ireton RC, Reynolds AB: A core function for p120-catenin in cadherin turnover. **J Cell Biol 163:**525-534, 2003
54. De Braekeleer E, Ferec C, De Braekeleer M: RUNX1 translocations in malignant hemopathies. **Anticancer Res 29:**1031-1037, 2009
55. de Urquiza AM, Liu S, Sjoberg M, Zetterstrom RH, Griffiths W, Sjovall J, et al: Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. **Science 290:**2140-2144, 2000
56. Deeb KK, Trump DL, Johnson CS: Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. **Nat Rev Cancer 7:**684-700, 2007
57. Dhawan P, Singh AB, Deane NG, No Y, Shiou S-R, Schmidt C, et al: Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. **J Clin Invest 115:**1765-1776, 2005
58. Díaz GD, Paraskeva C, Thomas MG, Binderup L, Hague A: Apoptosis is induced by the active metabolite of vitamin D_3 and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy. **Cancer Res 60:**2304-2312, 2000
59. Eckert JJ, Fleming TP: Tight junction biogenesis during early development. **Biochim Biophys Acta 1778:**717-728, 2008
60. Eckes B, Colucci-Guyon E, Smola H, Nodder S, Babinet C, Krieg T, et al: Impaired wound healing in embryonic and adult mice lacking vimentin. **J Cell Sci 113 (Pt 13):**2455-2462, 2000
61. Eckes B, Dogic D, Colucci-Guyon E, Wang N, Maniotis A, Ingber D, et al: Impaired mechanical stability, migration and contractile capacity in vimentin-deficient fibroblasts. **J Cell Sci 111 (Pt 13):**1897-1907, 1998
62. Eelen G, Gysemans C, Verlinden L, Vanoirbeek E, De Clercq P, Van Haver D, et al: Mechanism and potential of the growth-inhibitory actions of vitamin D and ana-logs. **Curr Med Chem 14:**1893-1910, 2007
63. Egeblad M, Werb Z: New functions for the matrix metalloproteinases in cancer progression. **Nat Rev Cancer 2:**161-174, 2002
64. Elias MC, Tozer KR, Silber JR, Mikheeva S, Deng M, Morrison RS, et al: TWIST is expressed in human gliomas and promotes invasion. **Neoplasia 7:**824-837, 2005
65. Eliseev RA, Dong YF, Sampson E, Zuscik MJ, Schwarz EM, O'Keefe RJ, et al: Runx2-mediated activation of the Bax gene increases osteosarcoma cell sensitivity to apoptosis. **Oncogene 27:**3605-3614, 2008
66. Elzagheid A, Kuopio T, Pyrhonen S, Collan Y: Lymph node status as a guide to selection of available prognostic markers in breast cancer: the clinical practice of the future? **Diagn Pathol 1:**41, 2006
67. Enomoto H, Enomoto-Iwamoto M, Iwamoto M, Nomura S, Himeno M, Kitamura Y, et al: Cbfa1 is a positive regulatory factor in chondrocyte maturation. **J Biol Chem 275:**8695-8702, 2000
68. Entz-Werle N, Stoetzel C, Berard-Marec P, Kalifa C, Brugiere L, Pacquement H, et al: Frequent genomic abnormalities at TWIST in human pediatric osteosarcomas. **Int J Cancer 117:**349-355, 2005
69. Erben RG, Soegiarto DW, Weber K, Zeitz U, Lieberherr M, Gniadecki R, et al: Deletion of deoxyribonucleic acid binding domain of the vitamin D receptor abrogates genomic and nongenomic functions of vitamin D. **Mol Endocrinol 16:**1524-1537, 2002
70. Esnard A, Esnard F, Faucher D, Gauthier F: Two rat homologues of human cystatin C. **FEBS Lett 236:**475-478, 1988

71. Evans SR, Nolla J, Hanfelt J, Shabahang M, Nauta RJ, Shchepotin IB: Vitamin D receptor expression as a predictive marker of biological behavior in human colorectal cancer. **Clin Cancer Res 4:**1591-1595, 1998
72. Evans SRT, Shchepotin EI, Young H, Rochon J, Uskokovic M, Shchepotin IB: 1,25-dihydroxyvitamin D_3 synthetic analogs inhibit spontaneous metastases in a 1,2-dimethylhydrazine-induced colon carcinogenesis model. **Int J Oncol 16:**1249-1254, 2000
73. Fanning AS, Little BP, Rahner C, Utepbergenov D, Walther Z, Anderson JM: The unique-5 and -6 motifs of ZO-1 regulate tight junction strand localization and scaffolding properties. **Mol Biol Cell 18:**721-731, 2007
74. Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. **Cell 61:**759-767, 1990
75. Fehrenbacher N, Jaattela M: Lysosomes as targets for cancer therapy. **Cancer Res 65:**2993-2995, 2005
76. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P: Estimates of the cancer incidence and mortality in Europe in 2006. **Ann Oncol 18:**581-592, 2007
77. Fernández-García NI, Pálmer HG, García M, González-Martín A, del Rio M, Barettino D, et al: 1α,25-Dihydroxyvitamin D_3 regulates the expression of *Id1* and *Id2* genes and the angiogenic phenotype of human colon carcinoma cells. **Oncogene 24:**6533-6544, 2005
78. Feskanich D, Ma J, Fuchs CS, Kirkner GJ, Hankinson SE, Hollis BW, et al: Plasma vitamin D metabolites and risk of colorectal cancer in women. **Cancer Epidemiol Biomarkers Prev 13:**1502-1508, 2004
79. Figueras J: Changing strategies in surgical treatment of colorectal liver metastases: state of the art. **Clin Transl Oncol 7:**229-231, 2005
80. Fodde R, Kuipers J, Rosenberg C, Smits R, Kielman M, Gaspar C, et al: Mutations in the *APC* tumour suppressor gene cause chromosomal instability. **Nat Cell Biol 3:**433-438, 2001
81. Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, Artavanis-Tsakonas S: Notch signals control the fate of immature progenitor cells in the intestine. **Nature 435:**964-968, 2005
82. Freije JP, Abrahamson M, Olafsson I, Velasco G, Grubb A, Lopez-Otin C: Structure and expression of the gene encoding cystatin D, a novel human cysteine proteinase inhibitor. **J Biol Chem 266:**20538-20543, 1991
83. Freije JP, Balbin M, Abrahamson M, Velasco G, Dalboge H, Grubb A, et al: Human cystatin D. cDNA cloning, characterization of the Escherichia coli expressed inhibitor, and identification of the native protein in saliva. **J Biol Chem 268:**15737-15744, 1993
84. Freije JP, Pendas AM, Velasco G, Roca A, Abrahamson M, Lopez-Otin C: Localization of the human cystatin D gene (CST5) to chromosome 20p11.21 by in situ hybridization. **Cytogenet Cell Genet 62:**29-31, 1993
85. Garland CF, Garland FC: Do sunlight and vitamin D reduce the likelihood of colon cancer? **Int J Epidemiol**, 2005
86. Garland CF, Garland FC: Do sunlight and vitamin D reduce the likelihood of colon cancer? **Int J Epidemiol 9:** 65-71, 1980
87. Gilles C, Polette M, Zahm JM, Tournier JM, Volders L, Foidart JM, et al: Vimentin contributes to human mammary epithelial cell migration. **J Cell Sci 112 (Pt 24):**4615-4625, 1999
88. Gilles C TE: The epithelial to mesenchymal transition and metastatic progression in carcinoma. **Breast J:**83-96, 1996
89. Gloushankova NA: Changes in regulation of cell-cell adhesion during tumor transformation. **Biochemistry (Mosc) 73:**742-750, 2008
90. Gocek E, Kielbinski M, Marcinkowska E: Activation of intracellular signaling pathways is necessary for an increase in VDR expression and its nuclear translocation. **FEBS Lett 581:**1751-1757, 2007
91. Gocheva V, Joyce JA: Cysteine cathepsins and the cutting edge of cancer invasion. **Cell Cycle 6:**60-64, 2007
92. Gocheva V, Zeng W, Ke D, Klimstra D, Reinheckel T, Peters C, et al: Distinct roles for cysteine cathepsin genes in multistage tumorigenesis. **Genes Dev 20:**543-556, 2006
93. Goeman JJ, van de Geer SA, de Kort F, van Houwelingen HC: A global test for groups of genes: testing association with a clinical outcome. **Bioinformatics 20:**93-99, 2004
94. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE: Tight junction proteins. **Prog Biophys Mol Biol 81:**1-44, 2003
95. González-Sancho JM, Larriba MJ, Ordóñez-Morán P, Pálmer HG, Muñoz A: Effects of 1alpha,25-dihydroxyvitamin D3 in human colon cancer cells. **Anticancer Res 26:**2669-2681, 2006

96. Gorham ED, Garland CF, Garland FC, Grant WB, Mohr SB, Lipkin M, et al: Optimal vitamin D status for colorectal cancer prevention: a quantitative meta analysis. **Am J Prev Med 32:**210-216, 2007
97. Goulet B, Baruch A, Moon NS, Poirier M, Sansregret LL, Erickson A, et al: A cathepsin L isoform that is devoid of a signal peptide localizes to the nucleus in S phase and processes the CDP/Cux transcription factor. **Mol Cell 14:**207-219, 2004
98. Grady WM, Myeroff LL, Swinler SE, Rajput A, Thiagalingam S, Lutterbaugh JD, et al: Mutational inactivation of transforming growth factor β receptor type II in microsatellite stable colon cancers. **Cancer Res 59:**320-324, 1999
99. Grant WB, Garland CF: Evidence supporting the role of vitamin D in reducing the risk of cancer. **J Intern Med 252:**178-179, 2002
100. Gregorieff A, Clevers H: Wnt signaling in the intestinal epithelium: from endoderm to cancer. **Genes Dev 19:**877-890, 2005
101. Groden J, Thliveris A, Samowitz W, Carlson M, Gelbert L, Albertsen H, et al: Identification and characterization of the familial adenomatous polyposis coli gene. **Cell 66:**589-600, 1991
102. Grubb A, Lofberg H: Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis. **Proc Natl Acad Sci U S A 79:**3024-3027, 1982
103. Guaita S, Puig I, Francí C, Garrido M, Domínguez D, Batlle E, et al: Snail induction of epithelial to mesenchymal transition in tumor cells is accompanied by *MUC1* repression and *ZEB1* expression. **J Biol Chem 277:**39209-39216, 2002
104. Gumbiner BM: Regulation of cadherin-mediated adhesion in morphogenesis. **Nat Rev Mol Cell Biol 6:**622-634, 2005
105. Halfon S, Ford J, Foster J, Dowling L, Lucian L, Sterling M, et al: Leukocystatin, a new Class II cystatin expressed selectively by hematopoietic cells. **J Biol Chem 273:**16400-16408, 1998
106. Hall A, Ekiel I, Mason RW, Kasprzykowski F, Grubb A, Abrahamson M: Structural basis for different inhibitory specificities of human cystatins C and D. **Biochemistry 37:**4071-4079, 1998
107. Hall A, Hakansson K, Mason RW, Grubb A, Abrahamson M: Structural basis for the biological specificity of cystatin C. Identification of leucine 9 in the N-terminal binding region as a selectivity-conferring residue in the inhibition of mammalian cysteine peptidases. **J Biol Chem 270:**5115-5121, 1995
108. Halline AG, Davidson NO, Skarosi SF, Sitrin MD, Tietze C, Alpers DH, et al: Effects of 1,25-dihydroxyvitamin D_3 on proliferation and differentiation of Caco-2 cells. **Endocrinology 134:**1710-1717, 1994
109. Hansen CM, Binderup L, Hamberg KJ, Carlberg C: Vitamin D and cancer: effects of $1,25(OH)_2D_3$ and its analogs on growth control and tumorigenesis. **Front Biosci 6:**D820-848, 2001
110. Harper KD, Iozzo RV, Haddad JG: Receptors for and bioresponses to 1,25-dihydroxyvitamin D in a human colon carcinoma cell line (HT-29). **Metabolism 38:**1062-1069, 1989
111. Harriss DJ, Atkinson G, George K, Cable NT, Reilly T, Haboubi N, et al: Lifestyle factors and colorectal cancer risk (1): systematic review and meta-analysis of associations with body mass index. **Colorectal Dis 11:**547-563, 2009
112. Hartsock A, Nelson WJ: Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. **Biochim Biophys Acta 1778:**660-669, 2008
113. Haussler MR, Whitfield GK, Haussler CA, Hsieh J-C, Thompson PD, Selznick SH, et al: The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. **J Bone Miner Res 13:**325-349, 1998
114. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al: Identification of c-MYC as a target of the APC pathway. **Science 281:**1509-1512, 1998
115. Hendrix MJ, Seftor EA, Seftor RE, Trevor KT: Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasive behavior. **Am J Pathol 150:**483-495, 1997
116. Hermiston ML, Wong MH, Gordon JI: Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for nonautonomous regulation of cell fate in a self-renewing system. **Genes Dev 10:**985-996, 1996
117. Hirado M, Tsunasawa S, Sakiyama F, Niinobe M, Fujii S: Complete amino acid sequence of bovine colostrum low-Mr cysteine proteinase inhibitor. **FEBS Lett 186:**41-45, 1985
118. Hoek K, Rimm DL, Williams KR, Zhao H, Ariyan S, Lin A, et al: Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. **Cancer Res 64:**5270-5282, 2004

119. Holick MF: Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. **Am J Clin Nutr 80:**1678S-1688S, 2004
120. Holick MF: Vitamin D deficiency. **N Engl J Med 357:**266-281, 2007
121. Holick MF: Vitamin D: A millenium perspective. **J Cell Biochem 88:**296-307, 2003
122. Hotz B, Arndt M, Dullat S, Bhargava S, Buhr HJ, Hotz HG: Epithelial to mesenchymal transition: expression of the regulators snail, slug, and twist in pancreatic cancer. **Clin Cancer Res 13:**4769-4776, 2007
123. Huerta S, Irwin RW, Heber D, Go VLW, Koeffler HP, Uskokovic MR, et al: 1α,25-$(OH)_2D_3$ and its synthetic analogue decrease tumor load in the Apc^{min} mouse. **Cancer Res 62:**741-746, 2002
124. Huhtakangas JA, Olivera CJ, Bishop JE, Zanello LP, Norman AW: The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1α,25$(OH)_2$-vitamin D_3 in vivo and in vitro. **Mol Endocrinol 18:**2660-2671, 2004
125. Humphries A, Wright NA: Colonic crypt organization and tumorigenesis. **Nat Rev Cancer 8:**415-424, 2008
126. Iacopetta B: TP53 mutation in colorectal cancer. **Hum Mutat 21:**271-276, 2003
127. Ikenouchi J, Matsuda M, Furuse M, Tsukita S: Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. **J Cell Sci 116:**1959-1967, 2003
128. Inubushi T, Kakegawa H, Kishino Y, Katunuma N: Specific assay method for the activities of cathepsin L-type cysteine proteinases. **J Biochem 116:**282-284, 1994
129. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M: Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. **Nature 363:**558-561, 1993
130. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP: Summaries of Affymetrix GeneChip probe level data. **Nucleic Acids Res 31:**e15, 2003
131. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al: Exploration, normalization, and summaries of high density oligonucleotide array probe level data. **Biostatistics 4:**249-264, 2003
132. Irvine JW, Coombs GH, North MJ: Cystatin-like cysteine proteinase inhibitors of parasitic protozoa. **FEMS Microbiol Lett 75:**67-72, 1992
133. Iseki K, Tatsuta M, Uehara H, Iishi H, Yano H, Sakai N, et al: Inhibition of angiogenesis as a mechanism for inhibition by 1α,-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 of colon carcinogenesis induced by azoxymethane in Wistar rats. **Int J Cancer 81:**730-733, 1999
134. Isemura S, Saitoh E, Ito S, Isemura M, Sanada K: Cystatin S: a cysteine proteinase inhibitor of human saliva. **J Biochem 96:**1311-1314, 1984
135. Isemura S, Saitoh E, Sanada K: Characterization and amino acid sequence of a new acidic cysteine proteinase inhibitor (cystatin SA) structurally closely related to cystatin S, from human whole saliva. **J Biochem 102:**693-704, 1987
136. Isemura S, Saitoh E, Sanada K: Characterization of a new cysteine proteinase inhibitor of human saliva, cystatin SN, which is immunologically related to cystatin S. **FEBS Lett 198:**145-149, 1986
137. Jansen-Durr P, Meichle A, Steiner P, Pagano M, Finke K, Botz J, et al: Differential modulation of cyclin gene expression by MYC. **Proc Natl Acad Sci U S A 90:**3685-3689, 1993
138. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al: Cancer statistics, 2008. **CA Cancer J Clin 58:**71-96, 2008
139. Jiao W, Miyazaki K, Kitajima Y: Inverse correlation between E-cadherin and Snail expression in hepatocellular carcinoma cell lines *in vitro* and *in vivo*. **Br J Cancer 86:**98-101, 2002
140. Joyce JA, Hanahan D: Multiple roles for cysteine cathepsins in cancer. **Cell Cycle 3:**1516-1619, 2004
141. Kalluri R, Neilson EG: Epithelial-mesenchymal transition and its implications for fibrosis. **J Clin Invest 112:**1776-1784, 2003
142. Kalluri R, Weinberg RA: The basics of epithelial-mesenchymal transition. **J Clin Invest 119:**1420-1428, 2009
143. Kang Y, Massague J: Epithelial-mesenchymal transitions: twist in development and metastasis. **Cell 118:**277-279, 2004
144. Kawano Y, Kypta R: Secreted antagonists of the Wnt signalling pathway. **J Cell Sci 116:**2627-2634, 2003

145. Kawaura A, Takahashi A, Tanida N, Oda M, Sawada K, Sawada Y, et al: 1α-Hydroxyvitamin D_3 suppresses colonic tumorigenesis induced by repetitive intrarectal injection of N-methyl-N-nitrosourea in rats. **Cancer Lett 55:**149-152, 1990
146. Kedinger V, Sansregret L, Harada R, Vadnais C, Cadieux C, Fathers K, et al: p110 CUX1 homeodomain protein stimulates cell migration and invasion in part through a regulatory cascade culminating in the repression of E-cadherin and occludin. **J Biol Chem 284:**27701-27711, 2009
147. Khare S, Bissonnette M, Scaglione-Sewell B, Wali RK, Sitrin MD, Brasitus TA: 1,25-dihydroxyvitamin D_3 and TPA activate phospholipase D in Caco-2 cells: role of PKC-α. **Am J Physiol 276:**G993-G1004, 1999
148. Kim MS, Kondo T, Takada I, Youn MY, Yamamoto Y, Takahashi S, et al: DNA demethylation in hormone-induced transcriptional derepression. **Nature 461:**1007-1012, 2009
149. Kinzler KW, Nilbert MC, Su LK, Vogelstein B, Bryan TM, Levy DB, et al: Identification of FAP locus genes from chromosome 5q21. **Science 253:**661-665, 1991
150. Kioulafa M, Balkouranidou I, Sotiropoulou G, Kaklamanis L, Mavroudis D, Georgoulias V, et al: Methylation of cystatin M promoter is associated with unfavorable prognosis in operable breast cancer. **Int J Cancer**, 2009
151. Klucky B, Mueller R, Vogt I, Teurich S, Hartenstein B, Breuhahn K, et al: Kallikrein 6 induces E-cadherin shedding and promotes cell proliferation, migration, and invasion. **Cancer Res 67:**8198-8206, 2007
152. Kobayashi H, Sagara J, Kurita H, Morifuji M, Ohishi M, Kurashina K, et al: Clinical significance of cellular distribution of moesin in patients with oral squamous cell carcinoma. **Clin Cancer Res 10:**572-580, 2004
153. Koblinski JE, Ahram M, Sloane BF: Unraveling the role of proteases in cancer. **Clin Chim Acta 291:**113-135, 2000
154. Koli K, Keski-Oja J: 1alpha,25-dihydroxyvitamin D3 and its analogues down-regulate cell invasion-associated proteases in cultured malignant cells. **Cell Growth Differ 11:**221-229, 2000
155. Kominsky SL, Argani P, Korz D, Evron E, Raman V, Garrett E, et al: Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. **Oncogene 22:**2021-2033, 2003
156. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al: Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. **Cell 89:**755-764, 1997
157. Krause G, Winkler L, Mueller SL, Haseloff RF, Piontek J, Blasig IE: Structure and function of claudins. **Biochim Biophys Acta 1778:**631-645, 2008
158. Krueger S, Haeckel C, Buehling F, Roessner A: Inhibitory effects of antisense cathepsin B cDNA transfection on invasion and motility in a human osteosarcoma cell line. **Cancer Res 59:**6010-6014, 1999
159. Krueger S, Kalinski T, Hundertmark T, Wex T, Kuster D, Peitz U, et al: Up-regulation of cathepsin X in Helicobacter pylori gastritis and gastric cancer. **J Pathol 207:**32-42, 2005
160. Krueger S, Kellner U, Buehling F, Roessner A: Cathepsin L antisense oligonucleotides in a human osteosarcoma cell line: effects on the invasive phenotype. **Cancer Gene Ther 8:**522-528, 2001
161. Kuester D, Lippert H, Roessner A, Krueger S: The cathepsin family and their role in colorectal cancer. **Pathol Res Pract 204:**491-500, 2008
162. Kuopio T, Kankaanranta A, Jalava P, Kronqvist P, Kotkansalo T, Weber E, et al: Cysteine proteinase inhibitor cystatin A in breast cancer. **Cancer Res 58:**432-436, 1998
163. Kurokawa M, Tanaka T, Tanaka K, Ogawa S, Mitani K, Yazaki Y, et al: Overexpression of the AML1 proto-oncoprotein in NIH3T3 cells leads to neoplastic transformation depending on the DNA-binding and transactivational potencies. **Oncogene 12:**883-892, 1996
164. Kwok WK, Ling MT, Lee TW, Lau TC, Zhou C, Zhang X, et al: Up-regulation of TWIST in prostate cancer and its implication as a therapeutic target. **Cancer Res 65:**5153-5162, 2005
165. Lah TT, Kokalj-Kunovar M, Strukelj B, Pungercar J, Barlic-Maganja D, Drobnic-Kosorok M, et al: Stefins and lysosomal cathepsins B, L and D in human breast carcinoma. **Int J Cancer 50:**36-44, 1992
166. Lah TT, Kos J, Blejec A, Frkovic-Georgio S, Golouh R, Vrhovec II, et al: The Expression of Lysosomal Proteinases and Their Inhibitors in Breast Cancer: Possible Relationship to Prognosis of the Disease. **Pathol Oncol Res 3:**89-99, 1997

167. Lappe JM, Travers-Gustafson D, Davies KM, Recker RR, Heaney RP: Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. **Am J Clin Nutr 85:**1586-1591, 2007
168. Larriba MJ, Martín-Villar E, García JM, Pereira F, Peña C, de Herreros AG, et al: Snail2 cooperates with Snail1 in the repression of vitamin D receptor in colon cancer. **Carcinogenesis 30:**1459-1468, 2009
169. Larriba MJ, Muñoz A: SNAIL *vs* vitamin D receptor expression in colon cancer: therapeutics implications. **Br J Cancer 92:**985-989, 2005
170. Laterza OF, Price CP, Scott MG: Cystatin C: an improved estimator of glomerular filtration rate? **Clin Chem 48:**699-707, 2002
171. Leontieva OV, Ionov Y: RNA-binding motif protein 35A is a novel tumor suppressor for colorectal cancer. **Cell Cycle 8:**490-497, 2009
172. Leung JY, Ehmann GL, Giangrande PH, Nevins JR: A role for Myc in facilitating transcription activation by E2F1. **Oncogene 27:**4172-4179, 2008
173. Levicar N, Kos J, Blejec A, Golouh R, Vrhovec I, Frkovic-Grazio S, et al: Comparison of potential biological markers cathepsin B, cathepsin L, stefin A and stefin B with urokinase and plasminogen activator inhibitor-1 and clinicopathological data of breast carcinoma patients. **Cancer Detect Prev 26:**42-49, 2002
174. Levicar N, Strojnik T, Kos J, Dewey RA, Pilkington GJ, Lah TT: Lysosomal enzymes, cathepsins in brain tumour invasion. **J Neurooncol 58:**21-32, 2002
175. Lien WH, Klezovitch O, Vasioukhin V: Cadherin-catenin proteins in vertebrate development. **Curr Opin Cell Biol 18:**499-506, 2006
176. Lin R, Nagai Y, Sladek R, Bastien Y, Ho J, Petrecca K, et al: Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D_3 analog EB1089 signaling on cell proliferation, differentiation, and immune system regulation. **Mol Endocrinol 16:**1243-1256, 2002
177. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al: Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. **Science 311:**1770-1773, 2006
178. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. **Methods 25:**402-408, 2001
179. Logan CY, Nusse R: The Wnt signaling pathway in development and disease. **Annu Rev Cell Dev Biol 20:**781-810, 2004
180. Lointier P, Wargovich MJ, Saez S, Levin B, Wildrick DM, Boman BM: The role of vitamin D_3 in the proliferation of a human colon cancer cell line *in vitro*. **Anticancer Res 7:**817-821, 1987
181. Lou J, Cao W, Bernardin F, Ayyanathan K, Rauscher IF, Friedman AD: Exogenous cdk4 overcomes reduced cdk4 RNA and inhibition of G1 progression in hematopoietic cells expressing a dominant-negative CBF - a model for overcoming inhibition of proliferation by CBF oncoproteins. **Oncogene 19:**2695-2703, 2000
182. Maes T, Barcelo A, Buesa C: Neuron navigator: a human gene family with homology to unc-53, a cell guidance gene from Caenorhabditis elegans. **Genomics 80:**21-30, 2002
183. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al: The nuclear receptor superfamily: the second decade. **Cell 83:**835-839, 1995
184. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al: The epithelial-mesenchymal transition generates cells with properties of stem cells. **Cell 133:**704-715, 2008
185. Maretzky T, Reiss K, Ludwig A, Buchholz J, Scholz F, Proksch E, et al: ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. **Proc Natl Acad Sci U S A 102:**9182-9187, 2005
186. Marra G, Boland CR: Hereditary nonpolyposis colorectal cancer: the syndrome, the genes, and historical perspectives. **J Natl Cancer Inst 87:**1114-1125, 1995
187. Marshman E, Booth C, Potten CS: The intestinal epithelial stem cell. **Bioessays 24:**91-98, 2002
188. Martin TA, Jiang WG: Loss of tight junction barrier function and its role in cancer metastasis. **Biochim Biophys Acta 1788:**872-891, 2009
189. McMichael AJ, Giles GG: Cancer in migrants to Australia: extending the descriptive epidemiological data. **Cancer Res 48:**751-756, 1988
190. Medici D, Hay ED, Olsen BR: Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. **Mol Biol Cell 19:**4875-4887, 2008
191. Meyerhardt JA, Mayer RJ: Systemic therapy for colorectal cancer. **N Engl J Med 352:**476-487, 2005

192. Mironchik Y, Winnard PT, Jr., Vesuna F, Kato Y, Wildes F, Pathak AP, et al: Twist overexpression induces in vivo angiogenesis and correlates with chromosomal instability in breast cancer. **Cancer Res 65:**10801-10809, 2005
193. Mitic LL, Van Itallie CM, Anderson JM: Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. **Am J Physiol Gastrointest Liver Physiol 279:**G250-254, 2000
194. Miyaki M, Konishi M, Tanaka K, Kikuchi-Yanoshita R, Muraoka M, Yasuno M, et al: Germline mutation of MSH6 as the cause of hereditary nonpolyposis colorectal cancer. **Nat Genet 17:**271-272, 1997
195. Mohamed MM, Sloane BF: Cysteine cathepsins: multifunctional enzymes in cancer. **Nat Rev Cancer 6:**764-775, 2006
196. Mohanam S, Jasti SL, Kondraganti SR, Chandrasekar N, Lakka SS, Kin Y, et al: Down-regulation of cathepsin B expression impairs the invasive and tumorigenic potential of human glioblastoma cells. **Oncogene 20:**3665-3673, 2001
197. Moon RT, Kohn AD, De Ferrari GV, Kaykas A: WNT and beta-catenin signalling: diseases and therapies. **Nat Rev Genet 5:**691-701, 2004
198. Morita M, Yoshiuchi N, Arakawa H, Nishimura S: CMAP: a novel cystatin-like gene involved in liver metastasis. **Cancer Res 59:**151-158, 1999
199. Murayama A, Takeyama K, Kitanaka S, Kodera Y, Kawaguchi Y, Hosoya T, et al: Positive and negative regulations of the renal 25-hydroxyvitamin D_3 1α-hydroxylase gene by parathyroid hormone, calcitonin, and 1α,25$(OH)_2D_3$ in intact animals. **Endocrinology 140:**2224-2231, 1999
200. Nakagawa T, Roth W, Wong P, Nelson A, Farr A, Deussing J, et al: Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. **Science 280:**450-453, 1998
201. Nakayama F, Semba S, Usami Y, Chiba H, Sawada N, Yokozaki H: Hypermethylation-modulated downregulation of claudin-7 expression promotes the progression of colorectal carcinoma. **Pathobiology 75:**177-185, 2008
202. Nathanson CM, Wasselius J, Wallin H, Abrahamson M: Regulated expression and intracellular localization of cystatin F in human U937 cells. **Eur J Biochem 269:**5502-5511, 2002
203. Nelson WJ, Nusse R: Convergence of Wnt, β-catenin, and cadherin pathways. **Science 303:**1483-1487, 2004
204. Newmark HL, Lipkin M, Maheshwari N: Colonic hyperplasia and hyperproliferation induced by a nutritional stress diet with four components of Western-style diet. **J Natl Cancer Inst 82:**491-496, 1990
205. Ng K, Meyerhardt JA, Wu K, Feskanich D, Hollis BW, Giovannucci EL, et al: Circulating 25-hydroxyvitamin d levels and survival in patients with colorectal cancer. **J Clin Oncol 26:**2984-2991, 2008
206. Ni J, Abrahamson M, Zhang M, Fernandez MA, Grubb A, Su J, et al: Cystatin E is a novel human cysteine proteinase inhibitor with structural resemblance to family 2 cystatins. **J Biol Chem 272:**10853-10858, 1997
207. Ni J, Fernandez MA, Danielsson L, Chillakuru RA, Zhang J, Grubb A, et al: Cystatin F is a glycosylated human low molecular weight cysteine proteinase inhibitor. **J Biol Chem 273:**24797-24804, 1998
208. Nieto MA: The SNAIL superfamily of zinc-finger transcription factors. **Nat Rev Mol Cell Biol 3:**155-166, 2002
209. Nishimura G, Manabe I, Tsushima K, Fujiu K, Oishi Y, Imai Y, et al: DeltaEF1 mediates TGF-beta signaling in vascular smooth muscle cell differentiation. **Dev Cell 11:**93-104, 2006
210. Nishimura T, Takeichi M: Remodeling of the adherens junctions during morphogenesis. **Curr Top Dev Biol 89:**33-54, 2009
211. Noe V, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, et al: Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. **J Cell Sci 114:**111-118, 2001
212. Norman AW: Sunlight, season, skin pigmentation, vitamin D, and 25-hydroxyvitamin D: integral components of the vitamin D endocrine system. **Am J Clin Nutr 67:**1108-1110, 1998
213. Norman AW, Mizwicki MT, Norman DP: Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. **Nat Rev Drug Discov 3:**27-41, 2004
214. Norman AW, Okamura WH, Bishop JE, Henry HL: Update on biological actions of 1α,25$(OH)_2$-vitamin D_3 (rapid effects) and 24R,25$(OH)_2$-vitamin D_3. **Mol Cell Endocrinol 197:**1-13, 2002

215. Nucci MR, Robinson CR, Longo P, Campbell P, Hamilton SR: Phenotypic and genotypic characteristics of aberrant crypt foci in human colorectal mucosa. **Hum Pathol 28:**1396-1407, 1997
216. Ohuchida K, Mizumoto K, Ohhashi S, Yamaguchi H, Konomi H, Nagai E, et al: Twist, a novel oncogene, is upregulated in pancreatic cancer: clinical implication of Twist expression in pancreatic juice. **Int J Cancer 120:**1634-1640, 2007
217. Olafsson I, Lofberg H, Abrahamson M, Grubb A: Production, characterization and use of monoclonal antibodies against the major extracellular human cysteine proteinase inhibitors cystatin C and kininogen. **Scand J Clin Lab Invest 48:**573-582, 1988
218. Omata F, Brown WR, Tokuda Y, Takahashi O, Fukui T, Ueno F, et al: Modifiable risk factors for colorectal neoplasms and hyperplastic polyps. **Intern Med 48:**123-128, 2009
219. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA: Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. **Cancer Res 68:**3645-3654, 2008
220. Ordóñez-Morán P, Larriba MJ, Pálmer HG, Valero RA, Barbáchano A, Dunach M, et al: RhoA-ROCK and p38MAPK-MSK1 mediate vitamin D effects on gene expression, phenotype, and Wnt pathway in colon cancer cells. **J Cell Biol 183:**697-710, 2008
221. Ordóñez-Morán P, Larriba MJ, Pendás-Franco N, Aguilera O, González-Sancho JM, Muñoz A: Vitamin D and cancer: an update of *in vitro* and *in vivo* data. **Front Biosci 10:**2723-2749, 2005
222. Oshima T, Kunisaki C, Yoshihara K, Yamada R, Yamamoto N, Sato T, et al: Reduced expression of the claudin-7 gene correlates with venous invasion and liver metastasis in colorectal cancer. **Oncol Rep 19:**953-959, 2008
223. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, et al: Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. **Cell 89:**765-771, 1997
224. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, et al: Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. **J Cell Physiol 143:**420-430, 1990
225. Pálmer HG, González-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, et al: Vitamin D_3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling. **J Cell Biol 154:**369-387, 2001
226. Pálmer HG, Larriba MJ, García JM, Ordónez-Morán P, Peña C, Peiró S, et al: The transcription factor SNAIL represses vitamin D receptor expression and responsiveness in human colon cancer. **Nat Med 10:**917-919, 2004
227. Pálmer HG, Sánchez-Carbayo M, Ordóñez-Morán P, Larriba MJ, Cordón-Cardó C, Muñoz A: Genetic signatures of differentiation induced by 1α,25-dihydroxyvitamin D_3 in human colon cancer cells. **Cancer Res 63:**7799-7806, 2003
228. Pan Q, Martell RE, O'Connell TD, Simpson RU: 1,25-Dihydroxyvitamin D3-regulated binding of nuclear proteins to a c-myc intron element. **Endocrinology 137:**4154-4160, 1996
229. Pan Q, Simpson RU: c-myc intron element-binding proteins are required for 1, 25-dihydroxyvitamin D3 regulation of c-myc during HL-60 cell differentiation and the involvement of HOXB4. **J Biol Chem 274:**8437-8444, 1999
230. Papapolychroniadis C: Environmental and other risk factors for colorectal carcinogenesis. **Tech Coloproctol 8 Suppl 1:**s7-9, 2004
231. Paredes R, Arriagada G, Cruzat F, Olate J, Van Wijnen A, Lian J, et al: The Runx2 transcription factor plays a key role in the 1alpha,25-dihydroxy Vitamin D3-dependent upregulation of the rat osteocalcin (OC) gene expression in osteoblastic cells. **J Steroid Biochem Mol Biol 89-90:**269-271, 2004
232. Paredes R, Arriagada G, Cruzat F, Villagra A, Olate J, Zaidi K, et al: Bone-specific transcription factor Runx2 interacts with the 1alpha,25-dihydroxyvitamin D3 receptor to up-regulate rat osteocalcin gene expression in osteoblastic cells. **Mol Cell Biol 24:**8847-8861, 2004
233. Peinado H, Olmeda D, Cano A: Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? **Nat Rev Cancer 7:**415-428, 2007
234. Peinado H, Portillo F, Cano A: Transcriptional regulation of cadherins during development and carcinogenesis. **Int J Dev Biol 48:**365-375, 2004
235. Pelengaris S, Khan M, Evan G: c-MYC: more than just a matter of life and death. **Nat Rev Cancer 2:**764-776, 2002

236. Peña C, García JM, Silva J, García V, Rodríguez R, Alonso I, et al: E-cadherin and vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: clinicopathological correlations. **Hum Mol Genet 14:**3361-3370, 2005
237. Perakyla M, Malinen M, Herzig KH, Carlberg C: Gene regulatory potential of nonsteroidal vitamin D receptor ligands. **Mol Endocrinol 19:**2060-2073, 2005
238. Perl A-K, Wilgenbus P, Dahl U, Semb H, Christofori G: A causal role for E-cadherin in the transition from adenoma to carcinoma. **Nature 392:**190-193, 1998
239. Perucho M: Cancer of the microsatellite mutator phenotype. **Biol Chem 377:**675-684, 1996
240. Perucho M: Microsatellite instability: the mutator that mutates the other mutator. **Nat Med 2:**630-631, 1996
241. Pinto D, Clevers H: Wnt, stem cells and cancer in the intestine. **Biol Cell 97:**185-196, 2005
242. Pinto D, Gregorieff A, Begthel H, Clevers H: Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. **Genes Dev 17:**1709-1713, 2003
243. Polette M, Gilles C, de Bentzmann S, Gruenert D, Tournier JM, Birembaut P: Association of fibroblastoid features with the invasive phenotype in human bronchial cancer cell lines. **Clin Exp Metastasis 16:**105-112, 1998
244. Poser I, Domínguez D, García de Herreros A, Varnai A, Buettner R, Bosserhoff AK: Loss of E-cadherin expression in melanoma cells involves up-regulation of the transcriptional repressor Snail. **J Biol Chem 276:**24661-24666, 2001
245. Postigo AA: Opposing functions of ZEB proteins in the regulation of the TGFbeta/BMP signaling pathway. **EMBO J 22:**2443-2452, 2003
246. Postigo AA, Depp JL, Taylor JJ, Kroll KL: Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. **Embo J 22:**2453-2462, 2003
247. Potten CS, Loeffler M: Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. **Development 110:**1001-1020, 1990
248. Powis G, Kirkpatrick DL: Thioredoxin signaling as a target for cancer therapy. **Curr Opin Pharmacol 7:**392-397, 2007
249. Powis G, Mustacich D, Coon A: The role of the redox protein thioredoxin in cell growth and cancer. **Free Radic Biol Med 29:**312-322, 2000
250. Pulukuri SM, Gorantla B, Knost JA, Rao JS: Frequent loss of cystatin E/M expression implicated in the progression of prostate cancer. **Oncogene 28:**2829-2838, 2009
251. Qiu J, Ai L, Ramachandran C, Yao B, Gopalakrishnan S, Fields CR, et al: Invasion suppressor cystatin E/M (CST6): high-level cell type-specific expression in normal brain and epigenetic silencing in gliomas. **Lab Invest 88:**910-925, 2008
252. Radtke F, Clevers H: Self-renewal and cancer of the gut: two sides of a coin. **Science 307:**1904-1909, 2005
253. Rajagopalan H, Nowak MA, Vogelstein B, Lengauer C: The significance of unstable chromosomes in colorectal cancer. **Nat Rev Cancer 3:**695-701, 2003
254. Rao R: Occludin phosphorylation in regulation of epithelial tight junctions. **Ann N Y Acad Sci 1165:**62-68, 2009
255. Rawlings ND, Barrett AJ: Evolution of proteins of the cystatin superfamily. **J Mol Evol 30:**60-71, 1990
256. Reichrath J, Lehmann B, Carlberg C, Varani J, Zouboulis CC: Vitamins as hormones. **Horm Metab Res 39:**71-84, 2007
257. Ricciardelli C, Sakko AJ, Ween MP, Russell DL, Horsfall DJ: The biological role and regulation of versican levels in cancer. **Cancer Metastasis Rev 28:**233-245, 2009
258. Risio M, Lipkin M, Newmark H, Yang K, Rossini FP, Steele VE, et al: Apoptosis, cell replication, and Western-style diet-induced tumorigenesis in mouse colon. **Cancer Res 56:**4910-4916, 1996
259. Rivenbark AG, Coleman WB: Epigenetic regulation of cystatins in cancer. **Front Biosci 14:**453-462, 2009
260. Ruiz-Ruiz C, Robledo G, Cano E, Redondo JM, López-Rivas A: Characterization of p53-mediated up-regulation of CD95 gene expression upon genotoxic treatment in human breast tumor cells. **J Biol Chem 278:**31667-31675, 2003
261. Sakakibara A, Furuse M, Saitou M, Ando-Akatsuka Y, Tsukita S: Possible involvement of phosphorylation of occludin in tight junction formation. **J Cell Biol 137:**1393-1401, 1997
262. Sanchez-Martinez R, Zambrano A, Castillo AI, Aranda A: Vitamin D-dependent recruitment of corepressors to vitamin D/retinoid X receptor heterodimers. **Mol Cell Biol 28:**3817-3829, 2008

263. Sancho E, Batlle E, Clevers H: Signaling pathways in intestinal development and cancer. **Annu Rev Cell Dev Biol 20:**695-723, 2004
264. Sansregret L, Goulet B, Harada R, Wilson B, Leduy L, Bertoglio J, et al: The p110 isoform of the CDP/Cux transcription factor accelerates entry into S phase. **Mol Cell Biol 26:**2441-2455, 2006
265. Schagdarsurengin U, Pfeifer GP, Dammann R: Frequent epigenetic inactivation of cystatin M in breast carcinoma. **Oncogene 26:**3089-3094, 2007
266. Schräder M, Nayeri S, Kahlen JP, Müller KM, Carlberg C: Natural vitamin D_3 response elements formed by inverted palindromes: polarity-directed ligand sensitivity of vitamin D_3 receptor-retinoid X receptor heterodimer-mediated transactivation. **Mol Cell Biol 15:**1154-1161, 1995
267. Segditsas S, Tomlinson I: Colorectal cancer and genetic alterations in the Wnt pathway. **Oncogene 25:**7531-7537, 2006
268. Shachaf CM, Gentles AJ, Elchuri S, Sahoo D, Soen Y, Sharpe O, et al: Genomic and proteomic analysis reveals a threshold level of MYC required for tumor maintenance. **Cancer Res 68:**5132-5142, 2008
269. Shah S, Islam MN, Dakshanamurthy S, Rizvi I, Rao M, Herrell R, et al: The molecular basis of vitamin D receptor and beta-catenin crossregulation. **Mol Cell 21:**799-809, 2006
270. Shridhar R, Zhang J, Song J, Booth BA, Kevil CG, Sotiropoulou G, et al: Cystatin M suppresses the malignant phenotype of human MDA-MB-435S cells. **Oncogene 23:**2206-2215, 2004
271. Singh S, Sadacharan S, Su S, Belldegrun A, Persad S, Singh G: Overexpression of vimentin: role in the invasive phenotype in an androgen-independent model of prostate cancer. **Cancer Res 63:**2306-2311, 2003
272. Sinha AA, Quast BJ, Wilson MJ, Fernandes ET, Reddy PK, Ewing SL, et al: Prediction of pelvic lymph node metastasis by the ratio of cathepsin B to stefin A in patients with prostate carcinoma. **Cancer 94:**3141-3149, 2002
273. Sinkkonen L, Malinen M, Saavalainen K, Väisänen S, Carlberg C: Regulation of the human *cyclin C* gene via multiple vitamin D_3-responsive regions in its promoter. **Nucleic Acids Res 33:**2440-2451, 2005
274. Sobrado VR, Moreno-Bueno G, Cubillo E, Holt LJ, Nieto MA, Portillo F, et al: The class I bHLH factors E2-2A and E2-2B regulate EMT. **J Cell Sci 122:**1014-1024, 2009
275. Sokol JP, Neil JR, Schiemann BJ, Schiemann WP: The use of cystatin C to inhibit epithelial-mesenchymal transition and morphological transformation stimulated by transforming growth factor-beta. **Breast Cancer Res 7:**R844-853, 2005
276. Sokol JP, Schiemann WP: Cystatin C antagonizes transforming growth factor beta signaling in normal and cancer cells. **Mol Cancer Res 2:**183-195, 2004
277. Sommers CL, Byers SW, Thompson EW, Torri JA, Gelmann EP: Differentiation state and invasiveness of human breast cancer cell lines. **Breast Cancer Res Treat 31:**325-335, 1994
278. Song J, Jie C, Polk P, Shridhar R, Clair T, Zhang J, et al: The candidate tumor suppressor CST6 alters the gene expression profile of human breast carcinoma cells: down-regulation of the potent mitogenic, motogenic, and angiogenic factor autotaxin. **Biochem Biophys Res Commun 340:**175-182, 2006
279. Sotiropoulou G, Anisowicz A, Sager R: Identification, cloning, and characterization of cystatin M, a novel cysteine proteinase inhibitor, down-regulated in breast cancer. **J Biol Chem 272:**903-910, 1997
280. Sporn MB RA, Goodman DS: **The Retinoids: Biology, Chemistry and Medicine., ed 2nd.** New York: Raven Press, 1994
281. Stenman G, Astrom AK, Roijer E, Sotiropoulou G, Zhang M, Sager R: Assignment of a novel cysteine proteinase inhibitor (CST6) to 11q13 by fluorescence in situ hybridization. **Cytogenet Cell Genet 76:**45-46, 1997
282. Stetler-Stevenson WG: The tumor microenvironment: regulation by MMP-independent effects of tissue inhibitor of metalloproteinases-2. **Cancer Metastasis Rev 27:**57-66, 2008
283. Strojan P, Budihna M, Smid L, Svetic B, Vrhovec I, Kos J, et al: Prognostic significance of cysteine proteinases cathepsins B and L and their endogenous inhibitors stefins A and B in patients with squamous cell carcinoma of the head and neck. **Clin Cancer Res 6:**1052-1062, 2000
284. Strojnik T, Zajc I, Bervar A, Zidanik B, Golouh R, Kos J, et al: Cathepsin B and its inhibitor stefin A in brain tumors. **Pflugers Arch 439:**R122-123, 2000

285. Strom DK, Nip J, Westendorf JJ, Linggi B, Lutterbach B, Downing JR, et al: Expression of the AML-1 oncogene shortens the G(1) phase of the cell cycle. **J Biol Chem 275:**3438-3445, 2000
286. Sutton ALM, MacDonald PN: Vitamin D: more than a "bone-a-fide" hormone. **Mol Endocrinol 17:**777-791, 2003
287. Takahashi H, Ibe M, Honma M, Ishida-Yamamoto A, Hashimoto Y, Iizuka H: 1,25-dihydroxyvitamin D(3) increases human cystatin A expression by inhibiting the Raf-1/MEK1/ERK signaling pathway of keratinocytes. **Arch Dermatol Res 295:**80-87, 2003
288. Takayama T, Katsuki S, Takahashi Y, Ohi M, Nojiri S, Sakamaki S, et al: Aberrant crypt foci of the colon as precursors of adenoma and cancer. **N Engl J Med 339:**1277-1284, 1998
289. Tangrea J, Helzlsouer K, Pietinen P, Taylor P, Hollis B, Virtamo J, et al: Serum levels of vitamin D metabolites and the subsequent risk of colon and rectal cancer in Finnish men. **Cancer Causes Control 8:**615-625, 1997
290. Taniyama T, Wanibuchi H, Salim EI, Yano Y, Otani S, Nishizawa Y, et al: Chemopreventive effect of 24R,25-dihydroxyvitamin D_3 in N, N'-dimethylhydrazine-induced rat colon carcinogenesis. **Carcinogenesis 21:**173-178, 2000
291. Thiery JP: Epithelial-mesenchymal transitions in tumour progression. **Nat Rev Cancer 2:**442-454, 2002
292. Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, et al: Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. **J Cell Biol 148:**189-202, 2000
293. Tinkle CL, Lechler T, Pasolli HA, Fuchs E: Conditional targeting of E-cadherin in skin: insights into hyperproliferative and degenerative responses. **Proc Natl Acad Sci U S A 101:**552-557, 2004
294. Toell A, Polly P, Carlberg C: All natural DR3-type vitamin D response elements show a similar functionality *in vitro*. **Biochem J 352:**301-309, 2000
295. Tong W-M, Bises G, Sheinin Y, Ellinger A, Genser D, Pötzi R, et al: Establishment of primary cultures from human colonic tissue during tumor progression: vitamin-D responses and vitamin-D-receptor expression. **Int J Cancer 75:**467-472, 1998
296. Turk B, Turk D, Turk V: Lysosomal cysteine proteases: more than scavengers. **Biochim Biophys Acta 1477:**98-111, 2000
297. Turk B, Turk V, Turk D: Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. **Biol Chem 378:**141-150, 1997
298. Turk V, Bode W: The cystatins: protein inhibitors of cysteine proteinases. **FEBS Lett 285:**213-219, 1991
299. Turk V, Brzin J, Longer M, Ritonja A, Eropkin M, Borchart U, et al: Protein inhibitors of cysteine proteinases. III. Amino-acid sequence of cystatin from chicken egg white. **Hoppe Seylers Z Physiol Chem 364:**1487-1496, 1983
300. Turk V, Turk B, Turk D: Lysosomal cysteine proteases: facts and opportunities. **EMBO J 20:**4629-4633, 2001
301. Tusher VG, Tibshirani R, Chu G: Significance analysis of microarrays applied to the ionizing radiation response. **Proc Natl Acad Sci U S A 98:**5116-5121, 2001
302. Usami Y, Chiba H, Nakayama F, Ueda J, Matsuda Y, Sawada N, et al: Reduced expression of claudin-7 correlates with invasion and metastasis in squamous cell carcinoma of the esophagus. **Hum Pathol 37:**569-577, 2006
303. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, et al: The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. **Cell 111:**241-250, 2002
304. van der Flier LG, Clevers H: Stem cells, self-renewal, and differentiation in the intestinal epithelium. **Annu Rev Physiol 71:**241-260, 2009
305. van Es JH, Clevers H: Notch and Wnt inhibitors as potential new drugs for intestinal neoplastic disease. **Trends Mol Med 11:**496-502, 2005
306. van Hengel J, van Roy F: Diverse functions of p120ctn in tumors. **Biochim Biophys Acta 1773:**78-88, 2007
307. van Roy FB, G.: The cell-cell adhesion molecule E-cadherin. **Cell Mol Life Sci 65:**3756-3788, 2008
308. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. **Genome Biol 3:**RESEARCH0034, 2002

309. Vandewalle B, Adenis A, Hornez L, Revillion F, Lefebvre J: 1,25-dihydroxyvitamin D_3 receptors in normal and malignant human colorectal tissues. **Cancer Lett 86:**67-73, 1994
310. Veena MS, Lee G, Keppler D, Mendonca MS, Redpath JL, Stanbridge EJ, et al: Inactivation of the cystatin E/M tumor suppressor gene in cervical cancer. **Genes Chromosomes Cancer 47:**740-754, 2008
311. Wactawski-Wende J, Kotchen JM, Anderson GL, Assaf AR, Brunner RL, O'Sullivan MJ, et al: Calcium plus vitamin D supplementation and the risk of colorectal cancer. **N Engl J Med 354:**684-696, 2006
312. Wang B, Sun J, Kitamoto S, Yang M, Grubb A, Chapman HA, et al: Cathepsin S controls angiogenesis and tumor growth via matrix-derived angiogenic factors. **J Biol Chem 281:**6020-6029, 2006
313. Wang L, Zhou X, Zhou T, Ma D, Chen S, Zhi X, et al: Ecto-5'-nucleotidase promotes invasion, migration and adhesion of human breast cancer cells. **J Cancer Res Clin Oncol 134:**365-372, 2008
314. Wang TT, Tavera-Mendoza LE, Laperriere D, Libby E, MacLeod NB, Nagai Y, et al: Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. **Mol Endocrinol 19:**2685-2695, 2005
315. Waterman ML: Lymphoid enhancer factor/T cell factor expression in colorectal cancer. **Cancer Metastasis Rev 23:**41-52, 2004
316. Werle B, Schanzenbacher U, Lah TT, Ebert E, Julke B, Ebert W, et al: Cystatins in non-small cell lung cancer: tissue levels, localization and relation to prognosis. **Oncol Rep 16:**647-655, 2006
317. Wex T, Buhling F, Wex H, Gunther D, Malfertheiner P, Weber E, et al: Human cathepsin W, a cysteine protease predominantly expressed in NK cells, is mainly localized in the endoplasmic reticulum. **J Immunol 167:**2172-2178, 2001
318. Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, et al: The genomic landscapes of human breast and colorectal cancers. **Science 318:**1108-1113, 2007
319. Xia L, Kilb J, Wex H, Li Z, Lipyansky A, Breuil V, et al: Localization of rat cathepsin K in osteoclasts and resorption pits: inhibition of bone resorption and cathepsin K-activity by peptidyl vinyl sulfones. **Biol Chem 380:**679-687, 1999
320. Xiao K, Oas RG, Chiasson CM, Kowalczyk AP: Role of p120-catenin in cadherin trafficking. **Biochim Biophys Acta 1773:**8-16, 2007
321. Xue L, Lipkin M, Newmark H, Wang J: Influence of dietary calcium and vitamin D on diet-induced epithelial cell hyperproliferation in mice. **J Natl Cancer Inst 91:**176-181, 1999
322. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al: Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. **Cell 117:**927-939, 2004
323. Yang K, Edelmann W, Fan K, Lau K, Leung D, Newmark H, et al: Dietary modulation of carcinoma development in a mouse model for human familial adenomatous polyposis. **Cancer Res 58:**5713-5717, 1998
324. Yasmin R, Williams RM, Xu M, Noy N: Nuclear import of the retinoid X receptor, the vitamin D receptor, and their mutual heterodimer. **J Biol Chem 280:**40152-40160, 2005
325. Yokoyama K, Kamata N, Hayashi E, Hoteiya T, Ueda N, Fujimoto R, et al: Reverse correlation of E-cadherin and Snail expression in oral squamous cell carcinoma cells in vitro. **Oral Oncol 37:**65-71, 2001
326. Yoshida CA, Komori T: Role of Runx proteins in chondrogenesis. **Crit Rev Eukaryot Gene Expr 15:**243-254, 2005
327. Yu AS, McCarthy KM, Francis SA, McCormack JM, Lai J, Rogers RA, et al: Knockdown of occludin expression leads to diverse phenotypic alterations in epithelial cells. **Am J Physiol Cell Physiol 288:**C1231-1241, 2005
328. Zaidi SK, Pande S, Pratap J, Gaur T, Grigoriu S, Ali SA, et al: Runx2 deficiency and defective subnuclear targeting bypass senescence to promote immortalization and tumorigenic potential. **Proc Natl Acad Sci U S A 104:**19861-19866, 2007
329. Zajchowski DA, Bartholdi MF, Gong Y, Webster L, Liu HL, Munishkin A, et al: Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells. **Cancer Res 61:**5168-5178, 2001
330. Zanello LP, Norman AW: Rapid modulation of osteoblast ion channel responses by 1α,25$(OH)_2$-vitamin D_3 requires the presence of a functional vitamin D nuclear receptor. **Proc Natl Acad Sci U S A 101:**1589-1594, 2004

331. Zeeuwen PL, Van Vlijmen-Willems IM, Jansen BJ, Sotiropoulou G, Curfs JH, Meis JF, et al: Cystatin M/E expression is restricted to differentiated epidermal keratinocytes and sweat glands: a new skin-specific proteinase inhibitor that is a target for cross-linking by transglutaminase. **J Invest Dermatol 116:**693-701, 2001
332. Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM, et al: Extrarenal expression of 25-hydroxyvitamin D_3-1α-hydroxylase. **J Clin Endocrinol Metab 86:**888-894, 2001
333. Zhang J, Shridhar R, Dai Q, Song J, Barlow SC, Yin L, et al: Cystatin m: a novel candidate tumor suppressor gene for breast cancer. **Cancer Res 64:**6957-6964, 2004
334. Zhang Z, Xie D, Li X, Wong YC, Xin D, Guan XY, et al: Significance of TWIST expression and its association with E-cadherin in bladder cancer. **Hum Pathol 38:**598-606, 2007
335. Zhao X, Feldman D: Regulation of vitamin D receptor abundance and responsiveness during differentiation of HT29 human colon cancer cells. **Endocrinology 132:**1808-1814, 1993
336. Zhao Y, Yan Q, Long X, Chen X, Wang Y: Vimentin affects the mobility and invasiveness of prostate cancer cells. **Cell Biochem Funct 26:**571-577, 2008
337. Zhu L, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, et al: Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. **Nature 457:**603-607, 2009

APPENDIX

Part of the work showed in this Thesis has been included in the following articles:

Silvia Álvarez-Díaz[*], Noelia Valle[*], José Miguel García, Cristina Peña, José M.P. Freije, Victor Quesada, Aurora Astudillo, Félix Bonilla, Carlos López-Otín and Alberto Muñoz. "Cystatin D is a candidate tumor suppressor gene induced by vitamin D in human colon cancer cells" *J. Clin. Invest.* 2009;119(8):2343-58.
[*] Equal contribution

Silvia Álvarez-Díaz, María Jesús Larriba, Carlos López-Otín and Alberto Muñoz. "Vitamin D: proteases, protease inhibitors and cancer" *Cell Cycle*. 2010;9(1).

Paloma Ordóñez-Morán, **Silvia Álvarez-Díaz**, Noelia Valle, María Jesús Larriba, Félix Bonilla and Alberto Muñoz. "The effects of 1,25-dihydroxyvitamin D3 on colon cancer cells depend on RhoA- ROCK-p38MAPK-MSK signaling" (submitted)

Other articles have been published during the work of this Thesis:

María Jesús Larriba[*], Noelia Valle[*], Hector G. Pálmer, Paloma Ordóñez-Morán, **Silvia Álvarez-Díaz**, Karl-Friedrich Becker, Carlos Gamallo, Antonio García de Herreros, José Manuel González-Sancho and Alberto Muñoz. "The inhibition of Wnt/β-catenin signalling by 1,25-dihydroxyvitamin D_3 is abrogated by Snail1 in human colon cancer cells" *Endocr Relat Cancer.* 2007;14(1):141-51.

María Jesús Larriba, Noelia Valle, **Silvia Álvarez** and Alberto Muñoz. "Vitamin D_3 and colorectal cancer". *Adv Exp Med Biol.* 2008;617:271-80.

Miguel F. Segura, Douglas Hanniford, Silvia Menendez, Lindsey Reavie, Xuanyi Zou, **Silvia Álvarez-Díaz**, Jan Zakrezewski, Elen Blochin, Amy Rose, Dusan Bogunovic, David Polsky, Jianjun Wei, Peng Lee, Ilana Belitskaya-Levy, Nina Bhardwaj, Iman Osman and Eva Hernando. "Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor" *Proc Natl Acad Sci*. 2009;106(6):1814-1819.

The active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$ Calcitriol) is a major regulator of gene expression in higher organisms. Many epidemiological and preclinical studies support a protective role of vitamin D against colon cancer, one of the most frequent and lethal neoplasias worldwide.

Protein abundance is an endpoint of gene expression that results from the balance between induction and degradation and is essential for adequate cell function. Proteins are degraded by proteases whose activity is in turn controlled by a number of endogenous protease inhibitors. Due to the crucial role of proteases in tumor development, the identification and study of their inhibitors and their role in tumor progression are of pressing interest.

Cystatin D is an inhibitor of several cysteine proteases of the cathepsin family. This protease inhibitor has an unknown biology and it had not been previously linked to cancer.

The results described in this Thesis show that $1\alpha,25(OH)_2D_3$ strongly induces the expression of cystatin D due to a direct transcriptional activation mediated by the vitamin D receptor (VDR). Ectopic cystatin D expression mimicked partially the effects of $1\alpha,25(OH)_2D_3$ in colon cancer cells while its silencing using shRNA attenuates these effects. In addition, exogenous cystatin D induced an adhesive phenotype and inhibited several transformation processes by antiproteolytic-dependent or -independent mechanisms. In human colorectal tumors, cystatin D expression decreased during tumorigenesis associated with tumor dedifferentiation and correlated with differentiation markers loss.

In summary, cystatin D acts as a tumor suppressor gene with unpredicted effects that may contribute to the antitumor action of $1\alpha,25(OH)_2D_3$ against colon cancer by mechanisms that are both dependent and independent of protease inhibition

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